Endothelial cell modulation of smooth muscle contraction

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Endothelial Cell Modulation of Smooth Muscle Contraction

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Abstract

1) Arterial diameter is regulated by the cells of the artery wall; the endothelial and smooth muscle cells. Experiments were designed to investigate the nature of endothelial cell activation and the mechanisms underlying endothelium-dependent relaxations of smooth muscle in isolated pressurized arteries.

2) In rat mesenteric and cremaster arteries, endothelial cell activation resulted in elevation and oscillation of endothelial cell free Ca^{2+} ($[Ca^{2+}]_i$). Oscillations were not synchronized between individual endothelial cells. In rat mesenteric arteries, sustained elevations of $[Ca^{2+}]_i$ were dependent on entry of extracellular Ca^{2+} with initial increases caused by the release of Ca^{2+} from intracellular stores. Control of $[Ca^{2+}]_i$ in rat mesenteric and cremaster vessels was apparently independent of membrane potential and may depend on store mediated Ca^{2+} entry.

3) Endothelium-dependent relaxation of rat mesenteric, rat cremaster and mouse mesenteric arteries involved both NO-dependent and NO-independent components. NO-independent components were sensitive to the combined blockade of both small (SK_{Ca}) and intermediate (IK_{Ca}) conductance Ca^{2+}-activated K^+ channels, blockade of neither channel individually was sufficient to fully inhibit NO-independent dilatations.

4) In rat cremaster arteries EDHF mediated relaxations were inhibited, but residual relaxations still occurred, when SK_{Ca} and IK_{Ca} were inhibited. The residual relaxations

were sensitive to either large (BK_{Ca}) conductance Ca^{2+}-activated K^+ channel inhibition, or cytochrome P-450 (CYP450) inhibition. Effects of BK_{Ca} or CYP450 inhibition could only be discerned following SK_{Ca} and IK_{Ca} blockade, indicating a secondary NO-independent dilatory mechanism which may be dependent on a CYP450 metabolite acting on BK_{Ca}.

5) Morphological studies identified cellular locations of K_{Ca} isoforms in mouse mesenteric arteries; SK_{Ca} and IK_{Ca} channels were confined to the endothelium, and were often co-localised with holes in the internal elastic lamina. Heterocellular coupling between the endothelium and smooth muscle was observed in rat cremaster arterioles.

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List of Abbreviations

17-ODYA	17-Octadecynoic acid
AA	Arachidonic Acid
ACh	Acetylcholine
BK_{Ca}	Large conductance Ca^{2+} activated K^+ channel
$[Ca^{2+}]_i$	Intracellular free Ca^{2+} concentration
CaM	Calmodulin
cAMP	Cyclic adenosine monophosphate
CCE	Capacitative Ca^{2+} entry
cGMP	Cyclic guanosine monophosphate
CPA	Cyclopiazonic acid
CRAC	Ca^{2+} release activated current
CYP450	Cytochrome P-450
DAG	Diacylglycerol
EC	Endothelial Cell
EDHF	Endothelium Derived hyperpolarizing factor
EET	epoxyeicosatrienoic acid
EDRF	Endothelium derived relaxing factor
HETE	Hydroxyeicosatetraenoic acid
IbTx	Iberiotoxin
IEL	Internal elastic lamina
IK_{Ca}	Intermediate conductance Ca^{2+} activated K^+ channel
IP_3	Inositol trisphosphate
IP_3R	Inositol trisphosphate recptor
K_{IR}	Inward rectifying K^+ channel
L-NAME	N-Nitro-L-Arginine Methyl Ester
M_3	Muscarinic receptor type 3
MEGJ	Myoendothelial gap junction
MLC	Myosin light chain
MLCK	Myosin light chain kinase
MLCP	Myosin light chain phosphatase
MSPPOH	*N*-methylsulfonyl-6-(2-propargyloxyphenyl)hexanamide
NK1	Neurokinin receptor type 1
NSCC	Non-selective cation channel
PAR	Protease activated receptor
PE	Phenylephrine
PGI_2	Prostacyclin
PIP_2	Phosphoinositol diphosphate
PKA	Protein kinase-A
PKC	Protein kinase-C
PKG	Protein kinase-G
PLA_2	Phospholipase-A_2
PLC	Phospholipase-C
PLP	Phospholipid

RhoK	*Rho* kinase
RyR	Ryanodine receptor
SERCA	Sacroplasmic endoplasmic reticulum ATPase
sGC	Soluble guanylate cyclase
SK_{Ca}	Small conductance Ca^{2+} activated K^{+} channel
SLIGRL	Leu-Iso-Gly-Arg-Leu
SMC	Smooth muscle cell
SOC	Store operated Ca^{2+} entry
TRAM-34	(1-[(2-chlorophenyl)diphenylmethyl]-1H-pyrazole)
TRP	Transient receptor potential
VOCC	Voltage operated Ca^{2+} channel
VR1	Vanilloid receptor type 1
VSM	Vascular smooth muscle

Journal Publications

McSHERRY I.N., SPITALER, M.M., TAKANO, H., DORA, K.A. (2005). Endothelial cell Ca^{2+} increases are independent of membrane potential in pressurized rat mesenteric arteries. *Cell Calcium*, **38,** (1), 23-33.

McSHERRY, I.N., SANDOW, S.L., CAMPBELL, W.B., FALCK, J.R., HILL, M.A., DORA, K.A. (2005). A role for heterocellular coupling and EETs in dilation of rat cremaster arteries. *Microcirculation, In Press.*

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Presented Research

Invited Oral Presentation

Role of endothelial cell K^+ channels in spreading dilatation.

Kim Dora, Hiromichi Takano, Michaela Spitaler, Iain McSherry, Chris Garland.

Experimental Biology, American Physiological Society; Cardiovascular Section: Vascular Communication and Coordinated Blood Distribution, Washington D.C., USA. 17^{th}-21^{st} April 2004.

Abstract; (2004) *FASEB J.*, Abstract 437.1.

Oral Presentations

Blockade of IK_{Ca} and SK_{Ca} channels inhibits EDHF-mediated vasodilatation, but not endothelial cell calcium changes, in isolated rat mesenteric arteries.

I.N. McSherry, C.J. Garland & K.A. Dora.

Australia & New Zealand Microcirculation Society, Progress in Microcirculation Research. 11th Australian and New Zealand Microcirculation Symposium. Fraser Island, Queensland, Australia. 11th-14th September 2003.

IK_{Ca} and SK_{Ca} endothelial cell hyperpolarization underlies vasodilatation to EDHF but does not modify calcium entry in rat isolated mesenteric arteries.

I.N. McSherry, C.J. Garland, K.A. Dora.

Physiological Society, Cambridge University, UK. 17th-18th December 2003.

Abstract; (2004) *J. Physiol.*, **555P,** C12.

Endothelium-dependent hyperpolarization in dilator responses of myogenically active mouse cremaster muscle arterioles.

S.J. Potocnik, C. Triggle, I. McSherry, K. Dora, N. Kotecha and M.A. Hill

New Developments in Resistance Artery Research, 8th International Symposium on Resistance Arteries (ISRA), Angers, France. June 20th- 23rd , 2004.

Abstract; (2004) *J. Vasc. Res.*, **41,** Suppl. 1, 4-15.

EDHF-like responses in rat cremaster muscle arterioles involve IK_{Ca} and SK_{Ca} channels, and CYP-450 metabolites.

I.N. McSherry, C.J. Garland, W. Campbell, J.R. Falck, M.A. Hill, K.A. Dora.

The 6th Asian Congress for Microcirculation (ACM'05), in conjunction with The 30th Annual Meeting of Japanese Society for Microcirculation (JSM'05), Tokyo, Japan. February 24th-26th, 2005.

Poster Presentations

Control of endothelial cell Ca^{2+} handling in rat mesenteric arteries.

I.N. McSherry, C.J. Garland, K.A. Dora.

British pharmacology Society, Joint Meeting with the Danish Society for Pharmacology and Toxicology, University of Bath, UK. July 6th-8th, 2004.

Abstract; (2004) *Br. J. Pharmacol., pa2,* **2** (2), 113P.

EDHF-like responses in rat cremaster muscle arterioles.

I.N. McSherry, C.J. Garland, W. Campbell, J.R. Falck, M.A. Hill, K.A. Dora.

Experimental Biology 2005 with XXXV International Congress of Physiological Sciences, San Diego, CA, USA. March 31st-April 6th, 2005.

EDHF-like responses in rat cremaster muscle arterioles.

I.N. McSherry, C.J. Garland, W. Campbell, J.R. Falck, M.A. Hill, K.A. Dora.

University of Bath Postgraduate Research Conference, University of Bath, Bath, UK. June 1st, 2005.

Chapter One

Introduction

1.1 The arterial system

The primary function of arteries is the supply of oxygen and nutrients to tissues in response to changes in demand. The control of tissue blood flow occurs primarily at the level of the small diameter arteries and arterioles, it is in these 'resistance vessels' that a significant increase in resistance against blood flow occurs. Resistance vessels are of high importance in determining the total vascular peripheral resistance, the resistance against which blood must flow. The peripheral resistance along with cardiac output, the volume of blood pumped per unit time, determines the arterial blood pressure, hence is a critical factor in a host of physiologies and pathophysiologies. A thorough understanding of the anatomy, physiology and pharmacology of resistance vessels is clearly required.

1.1.1 Arterial morphology

Vessels are comprised of three main concentric layers or 'tunics'; intima, media and adventitia (see Berne *et al.*, 1998). The tunica intima lines the lumen of vessels and is comprised solely of longitudinally aligned endothelial cells. Surrounding the tunica intima is a thin and fenestrated internal elastic lamina which separates the tunica intima from the tunica media. The tunica media is comprised of vascular smooth muscle cells which form rings of muscle around the blood vessels, and connective tissue. The outermost layer is the tunica adventitia which is comprised of collagen, elastic fibres, and limited amounts of fibroblasts. Perivascular nerves are associated with the tunica adventitia and typically do not penetrate the tunica media,

instead relying on diffusion of neurotransmitter from one of a series of variscosities on the nerve to the underlying smooth muscle and endothelial cells (for general reference see Berne *et al.*, 1998).

Physical connections often exist between cells in the vessel wall and are termed 'gap junctions' (figure 1.1) (for review see Saez *et al.*, 2003). Gap junctions may form an aqueous pore between adjoining cells of the same type, and indeed between endothelial cells and smooth muscle cells which are termed 'myoendothelial gap junctions'. Gap junctions are formed when two connexons from adjacent cells dock with each other. Connexons are a protein composed of an assembly of six subunits that are called connexins. Each complete gap junction comprises 12 connexins. Although ultrastructural evidence for gap junctions is not common such evidence exists for both homo- and hetero-cellular gap junctions in the arterial beds utilised in this study (rat mesenteric arteries (Sandow *et al.*, 2000a); mouse mesenteric arteries (Dora *et al.*, 2003); rat cremaster arteries (McSherry *et al.*, 2005a)).

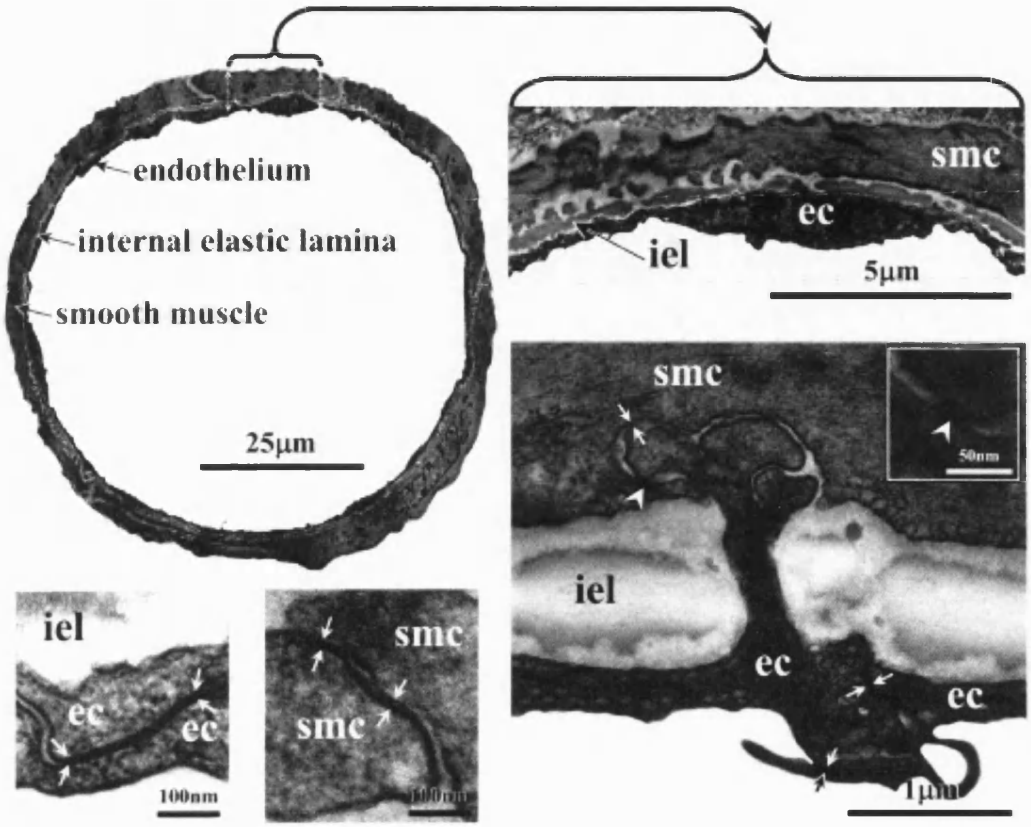

Figure 1.1; Morphology of a resistance artery. (Top left & top right) Cross-sections through a pressurized mouse cremaster artery demonstrate the typical composition of the wall of a resistance artery; an endothelial cell monolayer (ec), the internal elastic lamina (iel), and in this instance a single layer of smooth muscle cells (smc). (Bottom left) Homocellular gap junctions (arrowheads) may be observed between endothelial cells and smooth muscle cells and are characterised by a laminar morphology. (Bottom right) Typical hetero-cellular 'myoendothelial' gap junctions are formed from a projection of the endothelium passing through the internal elastic lamina to the vascular smooth muscle, (inset) zoom window of the laminar morphology.

(The work presented in this figure was performed in collaboration with Dr Shaun Sandow, University of Bath).

1.2 Control of vascular tone

Vessel diameter, and thus blood pressure, is ultimately determined by the level of tone present in vascular smooth muscle and hence the various pathways which affect constriction and/or relaxation.

Force generation in smooth muscle is the result of crossbridge cycling, essentially the interaction of myosin and actin filaments. In order for smooth muscle cross bridges to begin to cycle, the regulatory chain of smooth muscle myosin (MLC_{20}) must be phosphorylated. The major mechanism of MLC_{20} phosphorylation is myosin light chain kinase (MLCK). Dephosphorylation of MLC_{20}-P is mediated by smooth muscle myosin phosphatase (MLCP), the activity of which is regulated by signalling cascades involving both protein kinase-C (PKC) and *Rho*-kinase.

Constriction of smooth muscle may occur either by electromechanical coupling or pharmacomechanical coupling (Somlyo *et al.*, 1968), or indeed a combination of the two. In electromechanical coupling, intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) is increased following smooth muscle depolarization and the opening of voltage gated Ca^{2+} channels. Increases in $[Ca^{2+}]_i$ lead to Ca^{2+} binding to calmodulin, which then activates MLCK (Adelstein *et al.*, 1980). Pharmacomechanical coupling refers to constrictions occurring independently of membrane potential, i.e. from release of Ca^{2+} from intracellular stores or an alteration of the Ca^{2+} sensitivity of the contractile machinery, and can be demonstrated by differing levels of

$[Ca^{2+}]_i$ eliciting identical levels of constriction, or relaxations with minimal reductions in $[Ca^{2+}]_i$ (for review see Ratz *et al.*, 2005).

1.2.1 α_1-Adrenoceptor mediated arterial contraction

Physiologically, contraction of vascular smooth muscle is evoked via the release of neurotransmitters (noradrenaline, ATP and vasoactive peptides) from variscosities of perivascular nerves of the sympathetic nervous system. The ensuing contraction is predominantly due to the activation of smooth muscle α_1-adrenoceptors by noradrenaline. The synthetic selective α_1-adrenoceptor agonist, phenylephrine, is used throughout the experiments presented in this thesis, where preconstriction is required, to mimic the effects of noradrenaline. α_1-Adrenoceptors are $G_{q/11}$ coupled, their activation resulting in the hydrolysis of phosphatidylinositol, via the action of phospholipase-C, to inositol trisphosphate (IP_3) and diacylglycerol (DAG).

In brief, IP_3 activates IP_3 receptors located on the sarcoplasmic reticulum, leading to Ca^{2+} release from the sarcoplasmic reticulum. This Ca^{2+} can cause 'calcium induced calcium release' - the further release of Ca^{2+} from the sarcoplasmic reticulum through the Ca^{2+}-sensitive activation of IP_3 receptors and ryanodine receptors (for review see Berridge, 2002). The release of Ca^{2+} from intracellular stores can activate calcium sensitive chloride channels, resulting in chloride ion efflux and/or the activation of Na^+/Ca^{2+}-ATPase, both processes resulting in depolarization of the smooth muscle cell. However, the precise mechanisms remain to be elucidated given the lack of selective inhibitors (for review see Leblanc *et al.*, 2005). Smooth

muscle depolarization causes the opening of voltage gated L-type Ca^{2+} channels, causing influx of Ca^{2+} into the cell.

The contribution of DAG to the contraction process is uncertain at this time. However DAG is known to increase the activity of protein kinase C (PKC), and thus controls the phosphorylation of serine and threonine residues of a variety of intracellular proteins (Nishizuka, 1984; Nishizuka, 1986). PKC activators have been shown to increase the level of arteriolar tone without an increase in $[Ca^{2+}]_i$ (Gokina *et al.*, 1999), suggesting an effect on Ca^{2+} sensitivity, perhaps via inhibition of myosin light chain phosphatase, all of which makes PKC a plausible candidate for the actions of DAG (for review see Somlyo *et al.*, 2003). DAG has also been shown to activate transient receptor potential (TRP) channels both directly and indirectly (Hardie, 2003), providing a further mechanism whereby DAG could affect Ca^{2+} signalling (figure 1.2).

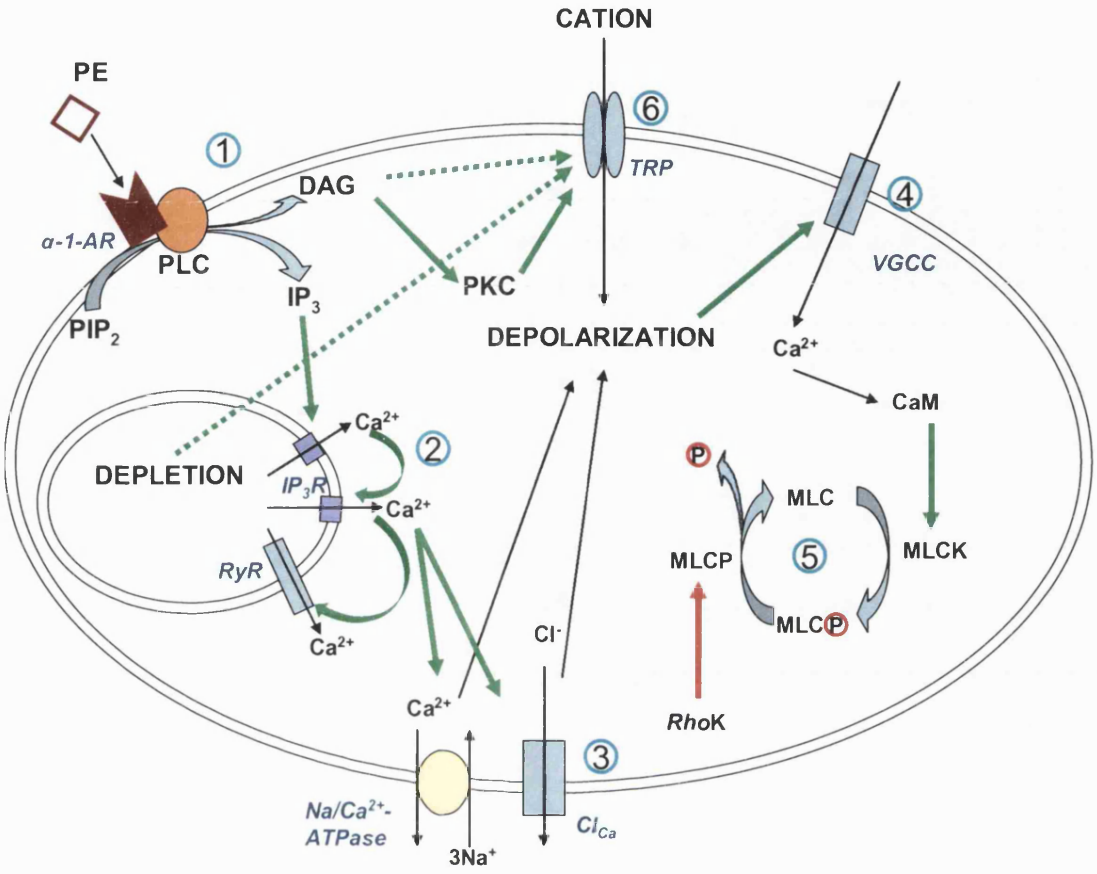

Figure 1.2; Mechanisms regulating smooth muscle contraction. 1) Binding of phenyephrine (PE) activates α_1-adrenoceptors (α-1-AR) leading to the formation of IP_3 and DAG via phospholipase-C. 2) IP_3 activates IP_3-receptors (IP_3R) on the sarcoplasmic reticulum, Ca^{2+} released from the sarcoplasmic reticulum may induce further Ca^{2+} release through both IP_3R and ryanodine receptors (RyR). 3) Intracellular Ca^{2+} increases may activate Ca^{2+}-activated chloride channels (Cl_{Ca}), and the Na^+/Ca^{2+}-ATPase, leading to smooth muscle depolarization. 4) Depolarization leads to Ca^{2+} entry through voltage gated Ca^{2+} channels (VGCC). 5) Ca^{2+} binds calmodlin (CaM), which activates myosin light chain kinase (MLCK) which phosphorylates myosin light chain (MLC). Myosin light chain phosphatase (MLCP) dephosphorylates MLC, its actions are inhibited by *Rho*kinase (*Rho*K). 6) TRP channel activation via store depletion or DAG, directly or indirectly via protein kinase-C may contribute to smooth muscle depolarization.

1.2.2 Pressure induced arterial contraction

Vascular preparations may respond to increased intraluminal pressure with a sustained constriction, and conversely a dilatation in response to a reduction in intraluminal pressure, a phenomenon known as the 'myogenic response'. Myogenic responses are independent of neural, metabolic and hormonal influences and have been demonstrated in arteries, venules, veins, lymphatics, and most prominently in arterioles (Davis *et al.*, 1999). Arteriolar myogenic responsiveness increases with decreasing vessel size down to the level of the second and third order arterioles (Davis, 1993), demonstrating an inverse relationship between vessel diameter and myogenic responsiveness. Whilst causative and modulatory influences involved in the generation and maintenance of myogenic tone are areas of active research, the physiological roles played by the myogenic response are less speculative; the myogenic response provides the flexibility of a local response to specific vascular needs (for example maintaining regular blood delivery in response to postural hypertension), generates a level of basal tone against which endogenous vasodilators may act, and also prevents the transmission of potentially damaging high pressure blood flow through capillary beds.

Increases in smooth muscle cell $[Ca^{2+}]_i$, monitored with Ca^{2+}-sensitive fluorescent probes, are seen with increased transmural pressure in myogenically active vessels (Meininger *et al.*, 1991). The necessity of extracellular Ca^{2+} entry for increases in smooth muscle Ca^{2+} seen during myogenic constrictions has been demonstrated by the lack of myogenic constriction following extracellular Ca^{2+} removal (Duling *et al.*, 1981; Uchida *et al.*, 1969), but the role, or otherwise, of intracellular Ca^{2+} stores in myogenic constriction is less clear. Evidence against a

prominent role for intracellular stores comes from the observation that whilst myogenic reactivity increases as vessel diameter diminishes (Davis, 1993) the importance of intracellular Ca^{2+} in constriction decreases with diminishing vessel diameter (Low *et al.*, 1996). In favour of a prominent role for intracellular Ca^{2+} stores in the generation of myogenic constrictions are; elevated IP_3 levels during myogenic constrictions (Narayanan *et al.*, 1994), attenuation of myogenic constrictions by phospholipase-C inhibition (Inscho *et al.*, 1998; Osol *et al.*, 1993), and the reduction of myogenic constrictions by agents which inhibit the sarcoplasmic reticulum Ca^{2+}-ATPase (Watanabe *et al.*, 1993). Regardless of the source of Ca^{2+}, the pressure-dependent increases in $[Ca^{2+}]_i$ bring about vascular smooth muscle contraction through binding and activation of MLCK, with subsequent phosphorylation of the MLC (Zou *et al.*, 1995; Zou *et al.*, 2000).

Intraluminal pressure has been shown to modulate membrane potential, with membrane depolarizations associated with increases in transmural pressure (Harder, 1984; Kotecha *et al.*, 2005). This depolarization opens voltage gated calcium channels and contraction ensues. Blockade of voltage gated Ca^{2+} channels (VGCC) blocks both myogenic constriction and Ca^{2+} increases, but not the smooth muscle cell membrane depolarization (Knot *et al.*, 1995; Setoguchi *et al.*, 1997) suggesting that whilst extracellular Ca^{2+} is a prerequisite for myogenic constriction it is not responsible for the membrane depolarization (McCarron *et al.*, 1997). Thus, either events which initiate membrane depolarization, or events which attenuate membrane hyperpolarization mechanisms, may play a part in generating the required membrane depolarization.

Increased transmural pressure may open stretch or tension activated non-selective cation channels, the existence of which has been demonstrated in vascular smooth muscle (Davis *et al.*, 1992a; Setoguchi *et al.*, 1997; Wu *et al.*, 2001). In isolated smooth muscle cells the relative ion permeability of these channels has been demonstrated to be $Na^+ > Ca^{2+}$ (Davis *et al.*, 1992b), thus Ca^{2+} influx via this pathway would be small in comparison to Na^+, indicating that stretch activation of these channels mainly contributes to membrane potential depolarization with subsequent opening of voltage gated Ca^{2+} channels, rather than being direct a route of Ca^{2+} influx. Store-operated Ca^{2+} entry (SOC) mechanisms may also contribute to membrane depolarization (see later section for description of SOC), whereby Ca^{2+} store depletion, which, analogously to stretch/tension activated channels, favours membrane depolarization with subsequent activation of voltage gated Ca^{2+} channels, rather than a direct influx of Ca^{2+} through non selective cation channels (Trepakova *et al.*, 2000; Trepakova *et al.*, 2001).

Vascular smooth muscle membrane hyperpolarization can occur via the opening of large-conductance Ca^{2+}-activated K^+ channels (BK_{Ca}), which are thought to be activated by Ca^{2+} "sparks" from ryanodine sensitive channels on the sarcoplasmic reticulum (Jaggar *et al.*, 1998). Given its inhibitory action on the BK_{Ca} channel (Zou *et al.*, 1996), 20-hydroxyeicosatetraenoic acid (20-HETE), a metabolite of cytochrome P-450 ω-hydroxylase, has been hypothesized to contribute to the generation and maintenance of myogenic tone (Looft-Wilson *et al.*, 2002). Recently, a complex mechanism concerning the involvement of 20-HETE in myogenic responses was postulated (Scotland *et al.*, 2004), whereby stretch and/or tension increases its release from vascular smooth muscle, which then acts on sensory C- and Aδ-fibres, which have been demonstrated to innervate various resistance vessels (Gulbenkian *et al.*, 1993). Following

sensory nerve activation with 20-HETE, increases in neuronal $[Ca^{2+}]_i$ lead to the vesicular release substance-P which acts on neurokinin-1 receptors located on vascular smooth muscle, which bring about muscle contraction (see figure 1.3).

Many other factors contributing to myogenic constrictions have been postulated, for example, pharmacomechanical constrictions mediated through inhibition of MLCK by either protein kinase-C and/or Rho-kinase associated mechanisms (Kitazawa *et al.*, 2000; Somlyo *et al.*, 2000). Furthermore, the products of PLC (IP_3 and DAG) have diverse vascular actions, IP_3 causes release of intracellular Ca^{2+}, whereas DAG activates protein kinase-C, is a source of arachidonic acid, and may activate NSCC.

A more thorough understanding of mechanisms involved in the generation and maintenance of myogenic tone will provide important insight into the autoregulation of blood pressure by resistance arteries, and may have important ramifications for pathologies in which altered myogenic responses have been demonstrated such as hypertension (Dunn *et al.*, 1998; Izzard *et al.*, 1996) and diabetes (Schofield *et al.*, 2002).

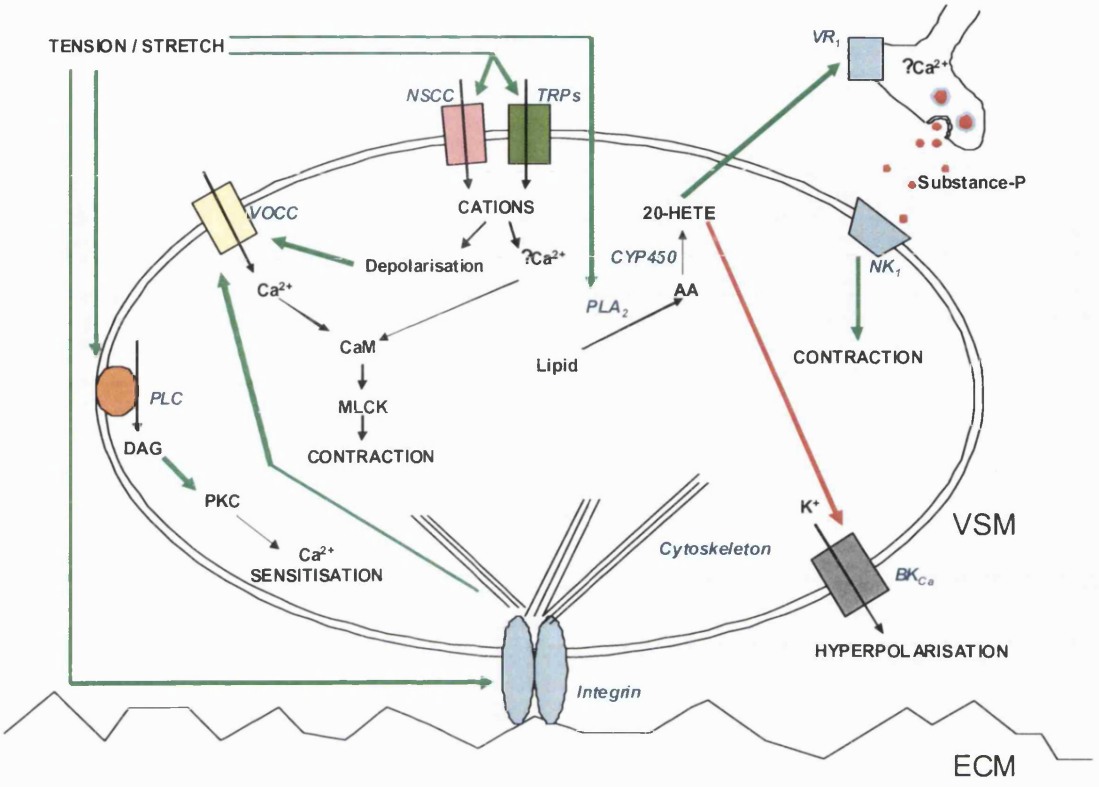

Figure 1.3; Mechanisms of pressure induced contraction. Increases in vessel tension and/or stretch, transduced to vascular smooth muscle cells (VSM) by integrins, can activate/increase a host of pathways involved in muscle constriction. ECM, extracellular matrix; PLC, phospholipase-C; DAG, diacylglycerol; PKC, protein kinase-C; VOCC, voltage operated Ca^{2+} channel; MLCK, myosin light chain kinase; NSCC, non-selective cation channel; TRPs, transient receptor potential channels; PLA_2, phospholipase-A_2; CYP450, cytochrome P-450; VR_1, vanilloid receptor 1; NK_1, neurokinin-1 receptor; AA, arachidonic acid.

1.3 Ca^{2+} signals and the regulation of Ca^{2+} activated K^+ channels

Increases in endothelial cell and smooth muscle cell $[Ca^{2+}]_i$ can exert opposing influences on blood vessel diameter, and the Ca^{2+}-signals arising in each cell type may also differ. In endothelial cells Ca^{2+} "waves" (a wave of Ca^{2+} which propagates across the cell) and "puffs" (small localised increases in Ca^{2+}) have been reported (McSherry *et al.*, 2005b, and Duza & Sarelius, 2004 respectively), although any specialised roles of these signals are yet to be ascertained. Three main types of Ca^{2+} signalling have been identified in vascular smooth muscle; global rises, Ca^{2+} waves and Ca^{2+} "sparks". Global increases in Ca^{2+} lead to constriction (Hai & Murphy, 1989) and can affect gene transcription (Cartin *et al.*, 2000). Ca^{2+} waves are thought to result from intracellular Ca^{2+} release through IP_3 and/or RyR (Boittin *et al.*, 1999), and may underly the rhythmical oscillations in vessel diameter seen during vasomotion (Peng *et al.*, 2001). Ca^{2+} sparks result from highly localised and large (10-100μM) releases of Ca^{2+} from intracellular Ca^{2+} stores (Nelson *et al.*, 1995), such localised signalling permits for microdomain signalling environments, i.e. Ca^{2+} sensitive process in the microdomain may be altered without affecting the global Ca^{2+} concentration.

SK_{Ca} and IK_{Ca} channels are voltage-insensitive and are activated by low concentrations of internal Ca^{2+} (<1.0μM) (for general review see Wei *et al.*, 2005). SK_{Ca} channels are sensitive to block by apamin, a protein isolated from bee venom, which distinguishes them from all other K_{Ca} channels. IK_{Ca} channels may be inhibited non-selectively by charybdotoxin, a protein from the venom of the Death Stalker scorpion (*Leiurus quinquestriatus)*, or selectively by the synthetic pound TRAM-34, a clotrimazole derivative (Wulff *et al.*, 2000). SK_{Ca} and IK_{Ca} do

not bind Ca^{2+} directly but rather detect Ca^{2+} by virtue of calmodulin, which is constitutively bound to the C-terminal region (Xia *et al.*, 1998), binding of Ca^{2+} to this calmodulin results in a conformational change in the channel which results in the generation of a unitary conductance of around 10pS.

The BK_{Ca} channel is sensitive to both Ca^{2+} and membrane potential. Under low-Ca^{2+} conditions, the BK_{Ca} behaves as a purely voltage-dependent K^+ channel. The voltage sensing mechanism of the BK_{Ca} is independent of Ca^{2+} binding, however, Ca^{2+} shifts many of the voltage dependent parameters to more negative voltages and allows the channel to function under a physiological range of membrane potentials (for review see Xia *et al.*, 2002). BK_{Ca} channels may be inhibited non-selectively by tetraethylammonium ions or charybdotoxin, or selectively by iberiotoxin, a protein isolated from the venom of the Red Indian scorpion (*Buthus tamulus*) (for a review of BK_{Ca} pharmacology see Ledoux *et al.* 2006).

There is emerging evidence that BK_{Ca} may be functionally linked to Ca^{2+} sparks. It is hypothesised the the Ca^{2+} sparks, emanating from the sarcoplasmic reticulum through ryanodine sensitive channels, are released at locations where the sarcoplasmic reticulum is in close proximity to the plasma membrane at regions expressing high levels of BK_{Ca} (for review see Jaggar *et al.*, 2000). More recently a more complicated model of this microdomain has been proposed whereby Ca^{2+} entry through TRPV4 channels enhances Ca^{2+} spark release (Earley *et al.*, 2005).

1.4 Endothelium dependent relaxation

In order to adequately control smooth muscle constriction a variety of relaxation mechanisms exist, many of which are endothelium dependent, thus making the endothelium an important regulator of vascular tone, local blood flow, and systemic blood pressure. Relaxation is elicited through either a reduction in smooth muscle $[Ca^{2+}]_i$, a decrease in the Ca^{2+} sensitivity of the contractile apparatus, or a combination of the two.

Initial studies into vasorelaxant factors released from the endothelium, revealed nitric oxide (NO) (Furchgott *et al.*, 1980; Palmer *et al.*, 1987), and prostacyclin (PGI_2) (Moncada *et al.*, 1976) to be endothelium derived relaxing factors (EDRFs). However, when endothelial nitric oxide synthase (eNOS) and cyclooxygenase (COX), the enzymes producing NO and PGI_2 respectively, are inhibited, many arteries still exhibit endothelium-dependent relaxations, particularly those in the resistance vasculature. These dilatations are associated with smooth muscle hyperpolarization, and thus decreased Ca^{2+} entry through voltage gated Ca^{2+} channels, characteristically involve endothelial cell Ca^{2+} activated K^+ channel (K_{Ca}) opening, and are due to the actions of a postulated 'endothelium-derived hyperpolarizing factor' (EDHF) (Taylor *et al.*, 1988). EDHF has been demonstrated to be of increasing importance in smaller diameter arteries and arterioles, probably reflecting the increased relative expression of voltage gated Ca^{2+} channels as diameter decreases (Shimokawa *et al.*, 1996).

Many groups dispute the identity and causes of EDHF, with potential EDHF candidates as diverse as K^+ (Edwards *et al.*, 1998), cytochrome P-450 (CYP450) epoxygenase products (Campbell *et al.*, 1996), anandamide (Randall *et al.*, 1996), hydrogen peroxide (Matoba *et al.*,

2000), *S*-nitrosothiols (Batenburg *et al.*, 2004a) and L-NAME insensitive NO (Chauhan *et al.*, 2000). Alternatively, it remains a possibility that endothelium dependent hyperpolarization is not due to a diffusible factor *per se*, rather it is caused by electrical coupling which exists between the endothelium and underlying smooth muscle cells via myoendothelial gap junctions (Emerson *et al.*, 2000; Sandow *et al.*, 2002; Xia *et al.*, 1995).

1.4.1 Nitric Oxide

First described as an EDRF (Furchgott *et al.*, 1980) and later indentified as nitric oxide, NO (Palmer *et al.*, 1987), NO diffuses from the endothelium to the underlying smooth muscle, where it activates soluble guanylate cyclase. Guanylate cyclase produces cyclic guanosine monophosphate (cGMP) from guanosine triphosphate, which in turn activates protein kinase G (PKG).

NO is released from the endothelium following either chemical (for example bradykinin) or mechanical (for example shear stress) stimulation. NO is synthesized by eNOS, during its conversion of L-arginine to L-citrulline. Several other routes of NO production have been suggested as an alternative source to nitric oxide synthase, including reactions between arginine and H_2O_2 (Nagase *et al.*, 1997), reduction of nitrite to NO (Modin *et al.*, 2001) and production of NO through cytochrome P450 (Karasu, 2000). There may also be stores of NO in vascular tissues, most likely as nitrosothiols, which are not linked to the action of nitric oxide synthase enzymes (Andrews *et al.*, 2003, Andrews *et al.*, 2002). The storage, and release, of NO in cells as nitrosothiols provides evidence that NO as well having autocrine and paracrine effects may also function as a blood/tissue borne hormone (Triggle *et al.*, 2003).

NO acts to relax vascular smooth muscle by two mechanisms; the reduction of smooth muscle $[Ca^{2+}]_i$, and the desensitization of the contractile machinery to Ca^{2+}, both through activation of the cGMP/PKG signalling pathway. Smooth muscle Ca^{2+} entry is reduced through voltage gated Ca^{2+} channel inhibition, both directly (Liu *et al.*, 1997), and indirectly through hyperpolarization of the smooth muscle cell membrane potential via an increase in the open probability of K_{Ca} channels (Archer *et al.*, 1994; Carrier *et al.*, 1997), and the activation of $3Na^+/2K^+$-ATPase located on the plasma membrane (Tamaoki *et al.*, 1997). Free intracellular Ca^{2+} is also reduced through PKG-mediated activation of plasma membrane Ca^{2+}-ATPase and the $3Na^+/Ca^{2+}$-ATPase (Furukawa *et al.*, 1988) leading to increased Ca^{2+} extrusion, futhermore there is increased uptake and decreased release of Ca^{2+} from intracellular stores through, respectively, increased activity of the sarcoplasmic reticulum Ca^{2+}-ATPase (Clapp *et al.*, 1991), and reduced activity of the IP_3 regulated channel (through channel phosphorylation and inhibition (Komalavilas *et al.*, 1994; Komalavilas *et al.*, 1996) and possibly through decreased IP_3 production due to inhibitory effects on phospholipase-C (Lincoln *et al.*, 1993)) (figure 1.4).

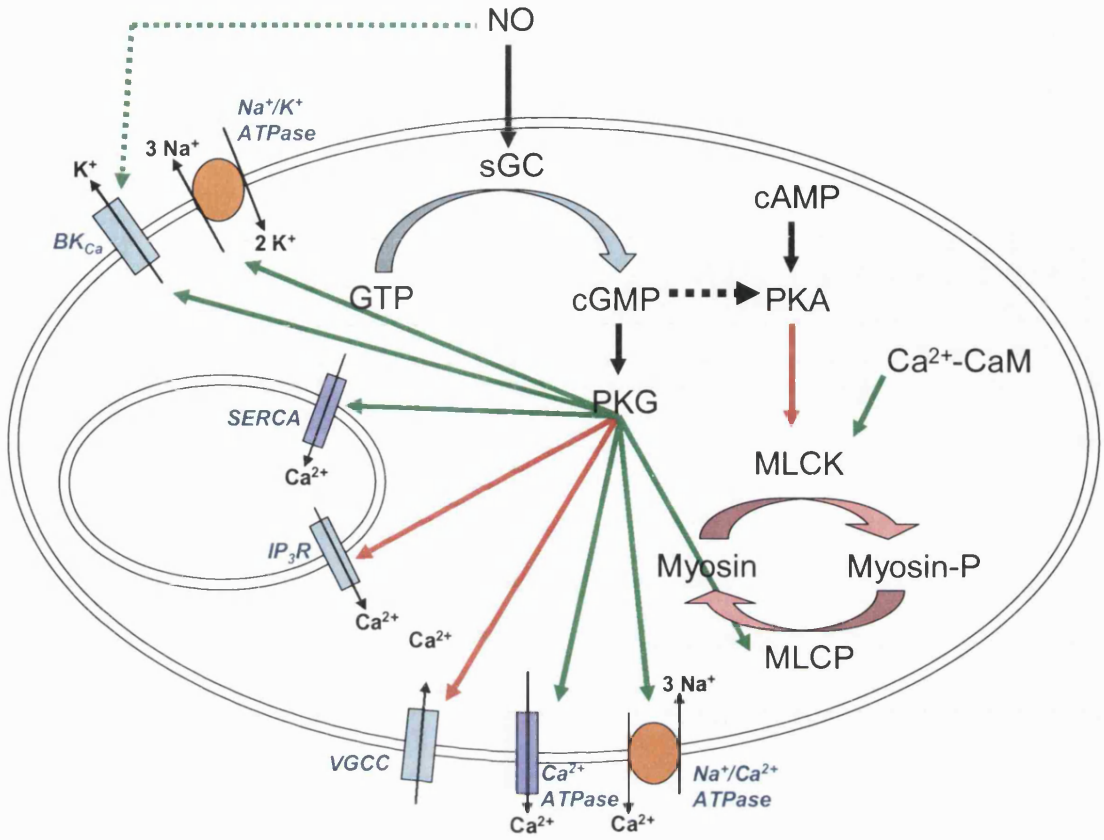

Figure 1.4; Current understanding of NO-mediated relaxation in vascular smooth muscle. NO inhibits both electromechanical and pharmacomechanical coupling. NO diffuses to underlying smooth muscle following its release from the endothelium, and activates soluble guanylate cyclase (sGC), leading to the production of cGMP from GTP, cGMP then activates protein kinase-G (PKG). PKG reduces intracellular Ca^{2+} through activation of; large conductance Ca^{2+}-activated K^+ channels (BK_{Ca}), sarcoplasmic-endoplasmic reticulum ATPase (SERCA), Na^+/Ca^{2+}-exchanger, Ca^{2+}-ATPase and Na^+/K^+-ATPase, and the inhibition of voltage gated Ca^{2+} channels (VGCC) and IP_3 gated channels (IP_3R). PKG decreases Ca^{2+} sensitivity of the contractile machinery via activation of myosin light chain phosphatase (MLCP), and possibly through cGMP cross activation of protein kinase A (PKA), leading to inhibition of myosin light chain kinase (MLCK), an enzyme which is typically activated by a Ca^{2+}-calmodulin (CaM) complex. NO may also directly activate BK_{Ca}.

1.4.2 Prostacyclin

PGI_2 was the first EDRF to be discovered (Moncada *et al.*, 1976), and is a product of cyclooxygenase metabolism of arachidonic acid. It is released from the endothelium and diffuses to the smooth muscle membrane where it binds a PGI_2 receptor, these receptors are G_s G-protein coupled receptors, therefore activation of these receptors results in activation of adenylate cyclase and subsequent generation of cyclic adenosine mono-phosphate (cAMP) from adenosine 5'-triphosphate. cAMP activates protein kinase-A, which can also phosphorylate, thus inactivate, myosin light chain kinase (de Lanerolle *et al.*, 1984). PGI_2 may also relax smooth muscle via hyperpolarization, via activation of ATP-sensitive K^+ channels (K_{ATP}) (Jackson *et al.*, 1993), or inwardly rectifying K^+ channels (K_{IR}) (Orie *et al.*, 2006). However, many endothelium-dependent agonist responses persist during cyclooxygenase inhibition, therefore PGI_2 is not considered to be a classical EDHF, especially in the peripheral circulation.

1.4.3 K^+ as EDHF

A breakthrough in the EDHF field occurred when it was demonstrated that EDHF responses were inhibited by the simultaneous presence of apamin (an inhibitor of small conductance calcium activated potassium channels, SK_{Ca}) and charybdotoxin (an inhibitor of intermediate and large conductance potassium channels, IK_{Ca} and BK_{Ca} respectively) but by neither inhibitor alone (Waldron *et al.*, 1994; Zygmunt *et al.*, 1996). It was also demonstrated that in the presence of apamin and iberiotoxin, a selective BK_{Ca} inhibitor, many EDHF responses persist (Zygmunt *et al.*, 1996). Strong evidence later emerged that the effect of apamin and

charybdotoxin combined was exerted on the vascular endothelium (Edwards *et al.*, 1998). This evidence taken together suggested that activation of IK_{Ca} and SK_{Ca} on the endothelium is essential for EDHF responses. Recently a highly selective inhibitor of the IK_{Ca} channel, 1-[(2-chlorophenyl) diphenylmethyl]-1*H*-pyrazole (TRAM-34), has been synthesized (Wulff *et al.*, 2000), which has allowed further confirmation of this hypothesis (Eichler *et al.*, 2003; Hinton *et al.*, 2003).

Typically, following endothelial cell activation, hyperpolarization of the endothelium occurs which is sensitive to SK_{Ca} and IK_{Ca} blockade (Hinton *et al.*, 2003), demonstrating it is due to the efflux of K^+ through K_{Ca} channels. This K^+ efflux from the endothelium has been shown to cause a small local increase in $[K^+]$ proximal to the underlying smooth muscle (Edwards *et al.*, 1998), which increases the outward current through K_{IR} present on vascular smooth muscle, and to increase the activity of Na^+/K^+-ATPases, which are electrogenic and are situated on vascular smooth muscle. The outcome of these effects is the hyperpolarization, and subsequent relaxation, of the vascular smooth muscle cells (figure 1.5).

Alternatively, it has been suggested that myoendothelial gap-junctions could play a critical role in EDHF mechanisms. K_{IR} have been described on the endothelium in some vessels (Crane *et al.*, 2003b), which, given the corresponding increase in extracellular $[K^+]$ would further amplify the hyperpolarization of the endothelium. Both electrical charge and low molecular weight dyes have been demonstrated to pass through myoendothelial gap junctions (for review see Coleman *et al.*, 2002), thus it has been hypothesized that the hyperpolarization of the endothelium may transfer to the muscle directly through gap junctions as an electrical event, negating the necessity of a putative factor. Indeed there is a consistent correlation

between the anatomical presence of myoendothelial gap junctions and endothelium dependent hyperpolarization of smooth muscle (Sandow *et al.*, 2000b; Sandow *et al.*, 2002). Several putative gap junction inhibitors have been mooted including glycerrhytinic acid and its derivatives (Guan *et al.*, 1996), and gap peptides, peptide sequences that exactly mimic those of sequences found in various regions of various connexins, the component proteins which combine to make a connexon (Chaytor *et al.*, 1997; Chaytor *et al.*, 1998), there are however concerns associated with the specificity of these agents (Tare *et al.*, 2002; Mather *et al.*, 2005).

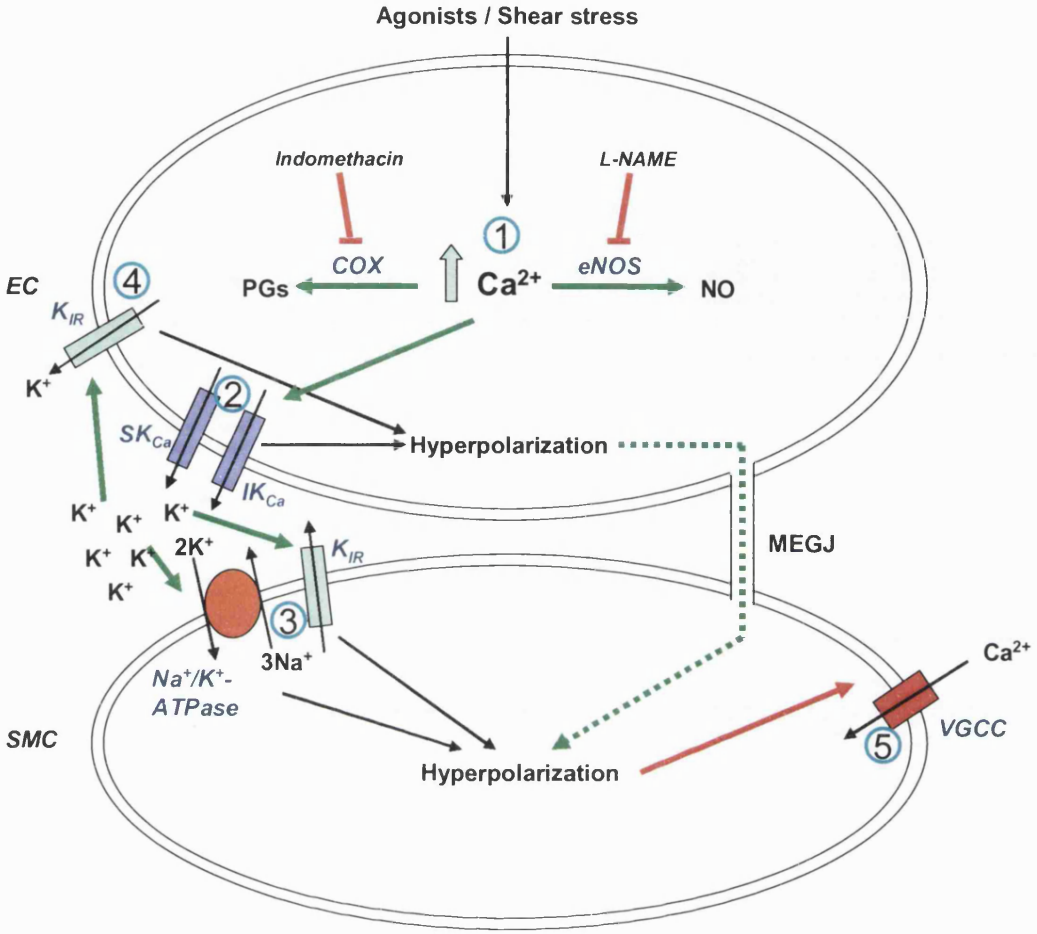

Figure 1.5; Involvement of endothelial K_{Ca} channels in EDHF responses. PGI_2- and NO-independent relaxations. Elevation of endothelial cell (EC) Ca^{2+} (1) activates Ca^{2+}-sensitive K^+ channels (K_{Ca}), resulting in K^+ efflux (2). (3) Elevated extracellular K^+ may activate inwardly-rectifying K^+ channels (K_{IR}) and Na^+/K^+-ATPases located on vascular smooth muscle cells (SMC), thereby hyperpolarizing the smooth muscle cell. (4) Alternatively, K_{IR} may be located on the endothelium, the hyperpolarization of which may transfer directly from the endothelium to smooth muscle via myoendothelial gap junctions (MEGJ). (5) Hyperpolarization of vascular smooth muscle reduces Ca^{2+} entry through voltage gated Ca^{2+} channels (VGCC), thereby driving relaxation.

1.4.4 Cytochrome P-450 as an EDHF-synthase

CYP450 enzymes are a group of haemoproteins whose Fe^{2+}/carbon monoxide complexes show an absorption spectrum with a maximum near 450nm. CYP450 is often described as the third pathway for arachidonic acid metabolism, following the establishment of arachidonic acid as a precursor of prostanoids and leukotrienes, through the actions of cyclooxygenase and lipoxygenase respectively. Following endothelial cell activation, increases in endothelial cell $[Ca^{2+}]_i$ increase the activity of phospholipase-A_2 leading to increased availability of arachidonic acid, a substrate of CYP450. CYP450 may metabolise arachidonic acid either via epoxygenation or by hydroxylation. With arachidonic acid as a substrate, epoxygenase activity can insert an epoxide residue between the carbons at positions 14-15, 11-12, 8-9 or 5-6, which leads to the formation of four regioisomers of the epoxyeicosatrienoic acids (EETs). Similarly with arachidonic acid as a substrate, the hydroxylase activity of CYP450 hydroxylase produces hydroxyeicosatetraenoic acids (HETEs), typically 20-HETE, although it may also produce 19-HETE.

Products of CYP450 have been hypothesized to be responsible for the EDHF response, specifically the products of the CYP450 epoxygenase, the EETs. EETs are postulated to activate BK_{Ca} channels located on vascular smooth muscle, with the application of exogenous EETs eliciting vessel relaxation and smooth muscle hyperpolarization, which is sensitive to the BK_{Ca} inhibitor iberiotoxin (Campbell *et al.*, 1996; McSherry *et al.*, 2005a). EETs may also exert effects on endothelial K_{Ca} channels (Edwards *et al.*, 2000; Weston *et al.*, 2005). An endothelial action of EETs would appear indicative of a facilatory role in the EDHF response

whereby EETs would increase endothelial cell hyperpolarization, rather than being an EDHF *per se*, or perhaps in addition to being a genuine EDHF. EETs have also been demonstrated to alter gap junctional communication in cultured cells, either directly, or via increases in cAMP (Popp *et al.*, 2002), providing further evidence of a modulatory role (figure 1.6).

The mechanism through which EETs affect K_{Ca} channels is currently defined in uncertain terms; some have postulated an EET receptor (Wong *et al.*, 1993), whilst other investigators believe that the action of EETs may be due to the activation of G_s proteins via ADP ribosylation (Fukao *et al.*, 2001; Li *et al.*, 1997; Li *et al.*, 1999; Li *et al.*, 2002). In addition to a postulated action on K_{Ca} channels, EETs have also been shown to activate TRPV4 channels in cultured human kidney cells (Vriens *et al.*, 2004; Watanabe *et al.*, 2003), activation of which results in Ca^{2+} entry, providing another possible mechanism whereby EETs may modulate vascular tone.

Conversely, 20-HETE, a product of CYP450 ω-hydroxylase, has been described as a vasoconstrictor, via inhibition of BK_{Ca} channels located on vascular smooth muscle (Zou *et al.*, 1996). This paints a complicated picture of CYP450 product function and is suggestive of strict structural requirements in order to modulate K_{Ca} activity, rather than, for example, a generic blocking or opening of the pore domain of K^+ channels. Furthermore, the exact expression patterns of the various CYP450 isoforms, and their tendency for epoxygenase versus hydroxylase activity, are critical to determine the vascular effect of CYP450 activation.

Increasingly the expression or otherwise of CYP450 isoforms can be linked to disease states, notably reduced expression in some forms of hypertension (for review see (Fleming, 2001)),

indicating that CYP450 and its products may have key roles in physiology and pathopyhsiology. Recently the synthesis of analogues of the EETs and HETEs has brought new and specific tools, both agonists and antagonists, to investigate the function of the CYP450 epoxygenase metabolites (Falck *et al.*, 2003; Gauthier *et al.*, 2002; Gauthier *et al.*, 2004; Yang *et al.*, 2005; Zhang *et al.*, 2001), which are helping to progress research in this area.

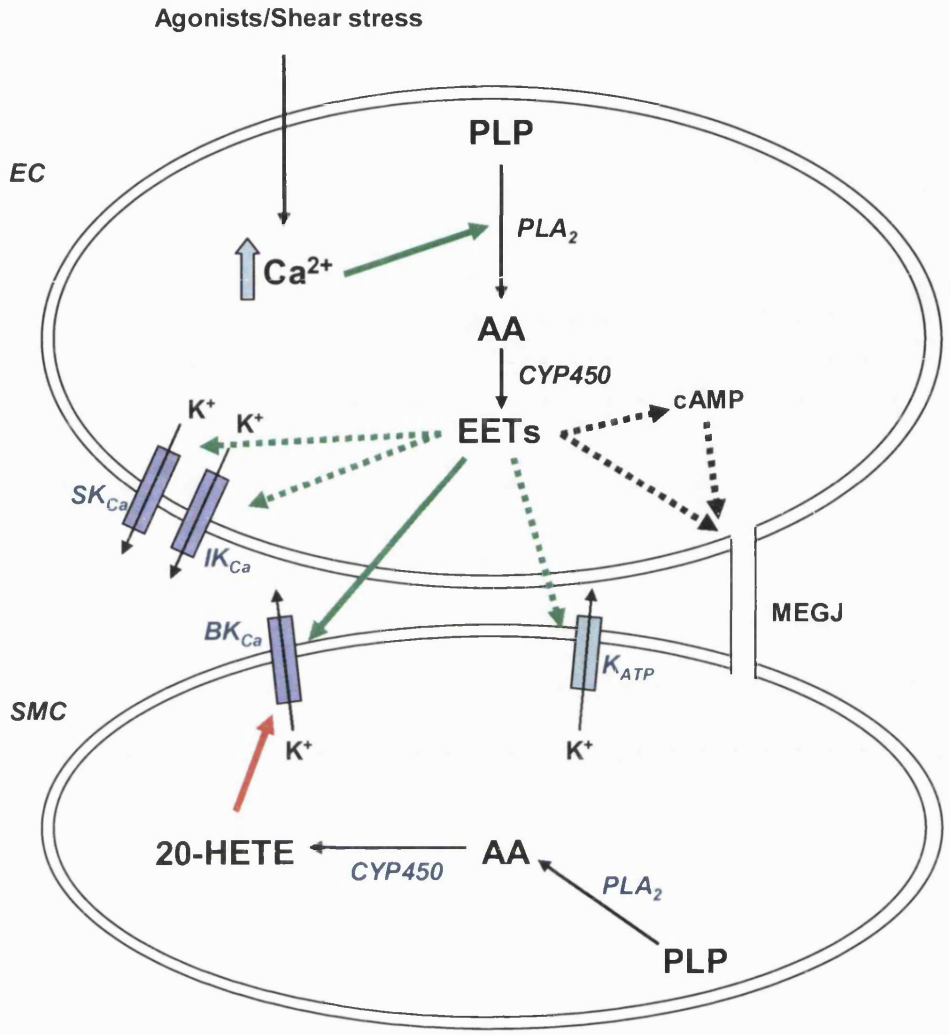

Figure 1.6; Involvement of cytochrome P-450 metabolites in EDHF responses. Increases in endothelial cell (EC) Ca^{2+} increase the production epoxyeicosatrienoic acids (EETs) by increasing the availability of the substrate (arachidonic acid, AA) for cytochrome P-450 (CYP450). EETs exert multiple effects, activation/increases are shown in green, inhibition/decreases are shown in red, postulated modulatory effects are shown by dashed lines. PLP; phospholipid, PLA_2; phospholipase-A_2, MEGJ; myoendothelial gap junction, SMC; smooth muscle cell.

1.4.5 H_2O_2 as an EDHF

Hydrogen peroxide (H_2O_2) is a reactive oxygen species which can be produced by the vascular endothelium and is a putative EDHF (Matoba *et al.*, 2000). The term reactive oxygen species describes a host of oxygen derived free- and non-free-radical molecules, including superoxide ($O_2^{\bullet-}$), hydroxyl radical ($^{\bullet}OH$) and the aforementioned H_2O_2. The endothelium has been demonstrated to produce significant amounts of free-radical species (Arroyo *et al.*, 1990; Brandes *et al.*, 1997), the sources of which are diverse and include; eNOS (Stroes *et al.*, 1998), CYP450 (Puntarulo *et al.*, 1998), cyclooxygenase (Sobey *et al.*, 1997) and NADPH oxidase (Mohazzab *et al.*, 1994).

H_2O_2 has been linked to a modulatory effect on potassium channels, a host of enzyme systems, and numerous pathways involved in Ca^{2+} homeostasis (for review see (Ellis *et al.*, 2003b)). Given the ubiquitous nature of H_2O_2 and its plethora of divergent effects, it is extremely difficult to state definitively whether it can function as an EDHF, indeed it has repeatedly been demonstrated to induce contractions of vascular preparations (Ellis *et al.*, 2003b). However, H_2O_2 has been linked to EDHF relaxations (Matoba *et al.*, 2002; Matoba *et al.*, 2000). Whilst H_2O_2 can be demonstrated to relax and hyperpolarize smooth muscle, EDHF mediated relaxations are not always inhibited by catalase (an enzyme responsible for H_2O_2 breakdown) (Beny *et al.*, 1991; Ellis *et al.*, 2003a; Fulton *et al.*, 1997; Hamilton *et al.*, 2001) and the onset of hyperpolarization to H_2O_2 differs from that induced by EDHF (Beny *et al.*, 1991), making it unlikely that H_2O_2 functions as a ubiquitous EDHF, although in certain situations its activity may be of important physiological relevance.

1.4.6 Alternative EDHF candidates

Cannabinoids, particularly anandamide, have been demonstrated to relax a host of arteries including cerebral (Ellis *et al.*, 1995), mesenteric (Jarai *et al.*, 1999; Plane *et al.*, 1997), and coronary arteries (Grainger *et al.*, 2001; White *et al.*, 2001), and the endothelium has been demonstrated to produce cannabinoids following administration of muscarinic agonists (Mechoulam *et al.*, 1998). However, anandamide is a substrate for CYP450 (Bornheim *et al.*, 1995), its metabolism leading to the production of a host of vasoactive compounds, clouding the precise role of anandamide as an EDHF. Furthermore investigations in rat mesenteric arteries demonstrate the site of action of anandamide, or its metabolites, as being BK_{Ca} channels, the proposed site of action of vasodilatory EETs, which further supports this hypothesis (Plane *et al.*, 1997). Cyclooxygenase inhibition has also been demonstrated to block the vasodilatatory actions of cannabinoids in cerebral arterioles (Ellis *et al.*, 1995).

L-NAME insensitive NO release has also been postulated as a potential EDHF (Chauhan *et al.*, 2003). This hypothesis is based on the ability of NO to elicit smooth muscle cell hyperpolarization (Cohen *et al.*, 1997; Tare *et al.*, 1990), and that the nitric oxide synthase inhibitor of choice, L-NAME, may not completely inhibit the release of NO following endothelial stimulation, with additional effects of NO-scavengers seen following L-NAME incubation in some vessels (Chauhan *et al.*, 2003; Ge *et al.*, 2000; Kemp *et al.*, 1997). It appears likely that whilst some of the residual relaxation and hyperpolarization that certain tissues exhibit in the presence of L-NAME (Chauhan *et al.*, 2003; Ge *et al.*, 2000) is dependent on NO, there remain large hyperpolarizations, and relaxations not linked to residual NO,

suggesting an alternative identity of EDHF (Vanheel *et al.*, 2000). Further evidence against NO as an EDHF comes from the demonstration of EDHF in eNOS deficient mice (Waldron *et al.*, 1999), and the observation that generation of superoxide anions, which avidly scavenge NO (Gryglewski *et al.*, 1986; Rubanyi *et al.*, 1986) does not affect relaxations which can be attributed to EDHF (Kaw *et al.*, 1999; Shimokawa *et al.*, 2004).

A limited number of reports have linked *S*-nitrosothiols with EDHF mechanisms (Batenburg *et al.*, 2004a; Batenburg *et al.*, 2004b), whereby *S*-nitrosothiols (a storage form of NO) activate endothelial SK_{Ca} and IK_{Ca} channels in a stereoselective fashion and also directly lead to increases in smooth muscle cGMP. This is a hypothesis in its infancy and more experiments are required to elucidate this mechanism.

1.5 Control of endothelial cell Ca^{2+}

As described, the endothelium is central to the control of vascular tone, through the release of vasoactive substances, and electrical coupling via myoendothelial gap junctions. In activated endothelial cells, an initial rise in $[Ca^{2+}]_i$ occurs through release from intracellular stores after which the Ca^{2+} supply is maintained from the extracellular space via either store depletion mediated entry and/or second messenger activation of Ca^{2+} entry, or a favourable electrochemical gradient. However, the relative contribution of the various Ca^{2+} signalling processes and Ca^{2+} influx channels in endothelial cells remains uncertain.

1.5.1 SOCs, CCEs, CRACs and TRPs

The first description of SOC (Casteels *et al.*, 1981), demonstrated that depletion of agonist-sensitive intracellular Ca^{2+} stores stimulated the rate of Ca^{2+} uptake from the extracellular solution in vascular smooth muscle cells. The entry pathway was shown to be tightly coupled to Ca^{2+} stores and blocked by manganese ions. Based on this description of SOC, the 'capacitative calcium entry' (CCE) concept was introduced, which proposed a mechanism by which activation of surface membrane receptors sustains Ca^{2+} entry into cells from the extracellular space (Putney, 1986; Putney, 1990). The current that was discovered in the first electrical measurement of SOC (Hoth *et al.*, 1992) was termed 'calcium-release-activated calcium current' (CRAC). However the molecular nature of SOC pathways, and SOC activation mechanisms were unknown. Candidate mechanisms include tyrosine phosphorylation (Fleming *et al.*, 1996), generation of a small molecular weight phosphorous containing compound (Randriamampita *et al.*, 1993), and activation via an arachidonic acid

metabolite (Rzigalinski *et al.*, 1999). The plasma membrane channel involved in SOC has been postulated to be a TRP cation channel, a cation channel originally described in *Drosophilla* eye (Hardie *et al.*, 1992).

Mammalian TRP proteins comprise of over 20 proteins which can aggregate to form ion channels. Recently a unified nomenclature of the TRP family proteins has been proposed based on homology (Montell *et al.*, 2002). Members of the TRP-related proteins are classified into three subfamilies; TRPC (C for canonical), TRPV (V for vanilloid) and TRPM (M for melastatin). Endothelial cells are known to express 6 of the 7 known TRPC members, with TRPC7 not yet reported (Chang *et al.*, 1997; Freichel *et al.*, 1999). Proteins from both the TRPV and TRPM sub-families have also been located on the endothelium and may also be involved in Ca^{2+} signalling (see Nilius, 2003).

TRP activation mechanisms are poorly understood and may vary between TRP members, for example TRPC3 and TRPC6 appear to be activated by DAG and by binding of polyunsaturated fatty acids (Hofmann *et al.*, 1999), whereas TRPC1 and TRPC4 appear to be activated by cell depletion (Brough *et al.*, 2001; Freichel *et al.*, 2001). Nearly all TRPC channels have been clearly linked with SOC mechanisms (for review see Nilius, 2003), presenting a seemingly complicated picture. It is difficult to categorically state which TRP-channels are involved in a given response given the lack of selective inhibitors of the various forms, and in the knowledge that TRPC proteins can assemble into homomeric or heteromeric ion channels (Hofmann *et al.*, 2002), a further level of complexity is added.

1.5.2 Endothelial cell hyperpolarization and Ca^{2+} entry

The activation of endothelial cells is known to be associated with an increase in $[Ca^{2+}]_i$ and in membrane hyperpolarization. As hyperpolarization enhances the driving force for Ca^{2+} entry (figure 1.7), and endothelial cells have been shown to lack voltage gated Ca^{2+} channels (Cannell *et al.*, 1989; Colden-Stanfield *et al.*, 1987), hyperpolarization of endothelial cells, will lead to a concommitant influx of Ca^{2+} into endothelial cells. The membrane potential of endothelial cells is thought to be largely controlled via potassium channels, including the K_{IR}, which are constitutively open, and K_{Ca} and K_{ATP}, which are activated during endothelial cell stimulation (Nilius *et al.*, 2001).

However, whilst the key role of membrane potential in endothelial cell Ca^{2+} entry is established in isolated or cultured cells (for example Luckhoff *et al.*, 1990; Schilling, 1989), reports investigating intact vessels are scarce. Recent reports utilizing whole vessel methodology (Ghisdal *et al.*, 2001; Cohen *et al.*, 2005; McSherry *et al.*, 2005b) suggest that Ca^{2+} entry into endothelial cells during maintained responses is independent of membrane potential, contradicting results from cultured and isolated cells.

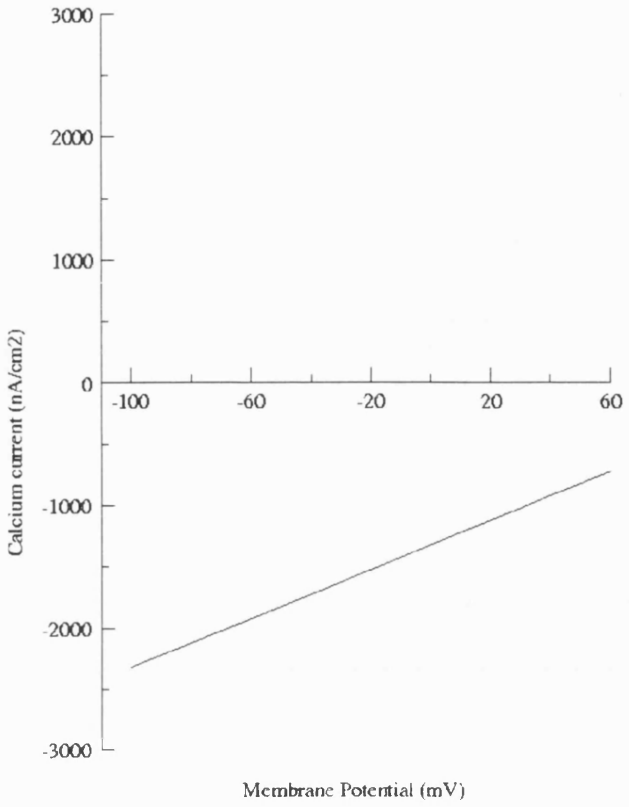

Figure 1.7; Membrane potential and Ca^{2+} entry driving forces. Taking the example of Ca^{2+} in endothelial cells at 37°C, and assuming extracellular and intracellular [Ca^{2+}] of 2mM and $1x10^{-7}$M respectively, then E_{Ca2+}=+132.2mV. Therefore a membrane hyperpolarization would increase the electrochemical gradient for Ca^{2+} entry by increasing the difference between the membrane potential and equilibrium potential.

1.6 Research Aims

The experiments presented in this thesis are designed to investigate the nature of endothelial cell activation, and the mechanisms underlying the vasodilatatory responses generated.

The activation of endothelial cell K_{Ca} channels is considered to be a critical step in EDHF generation. The specific isoforms responsible are hypothesized to be SK_{Ca} and IK_{Ca}, although this assumption is in part based on the use and effects of a non-selective drug, namely charybdotoxin, which is used to block IK_{Ca} channels. A novel drug, TRAM-34, with high IK_{Ca} selectivity was tested in order to confirm this hypothesis in rat mesenteric and cremaster, and mouse mesenteric arteries. Cellular location of K_{Ca} isoforms was also tested by immunohistological methods in the mouse mesenteric artery.

The driving force for agonist induced Ca^{2+} entry into endothelial cells has historically been presumed to arise due to the favourable electrochemical gradient generated by the membrane potential hyperpolarisation which typically accompanies agonist administration, however, recent evidence (Marrelli *et al.*, 2001) has implied that membrane potential may be of negligable importance in Ca^{2+} entry in this cell type. Both these hypotheses were tested in rat mesenteric and cremaster vessels.

The presence of more than one EDHF mechanism has been widely postulated (see Busse *et al.*, 2002). Particular attention has fallen on products of CYP450 enzymes as putative EDHFs. This hypothesis was tested further in rat cremaster arteries, a vessel which has previously been

linked with CYP450 dependent responses (Bakker & Sipkema, 1997). Experiments were also carried out to test the hypothesis that EDHF generation is dependent on a Ca^{2+} sensitive 'EDHF-synthase', and thus on endothelial cell $[Ca^{2+}]_i$, by monitoring the effects of inhibitors on vessel diameter and Ca^{2+}.

Chapter Two

Methods

2.1 Isobaric diameter recordings

The majority of the experiments presented in this thesis utilized pressure myography (see figure 2.1), a technique which enables the measurement of vessel diameter at a constant and precise transmural pressure, enables luminal perfusion of compounds, allows investigation of the effects of luminal flow, and which causes minimal disruption to the vessel wall.

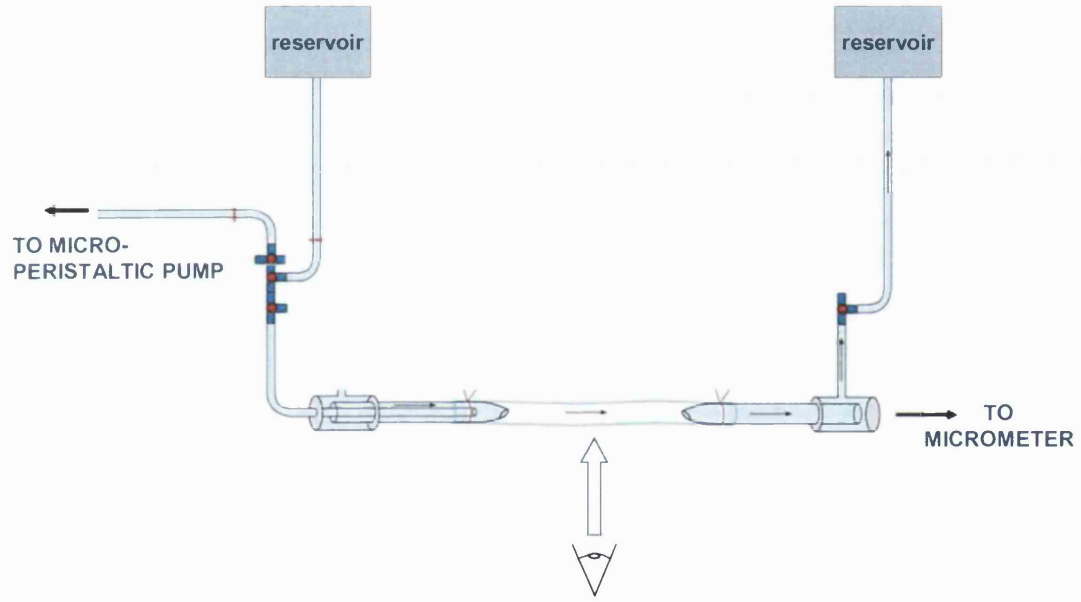

Figure 2.1; Diagrammatic representation of pressure myography. A length of artery is cannulated onto glass pipettes and is secured in place with silk ties. The luminal solution is gravity fed by buffer reservoirs, hence adjusting their height will adjust the intraluminal pressure. Luminal flow may be created by creating a height difference between the two reservoirs or through use of the microperistaltic pump.

2.2 RAT MESENTERIC STUDY

Male Wistar rats (200 - 250 g) were killed by cervical dislocation and exsanguination (Schedule 1 procedure; UK Animals (Scientific Procedures) Act 1986).

2.2.1 Pressure myography

The mesenteric arcade was removed and placed in chilled MOPS buffer (4°C) containing (mM): 145 NaCl, 4.7 KCl, 2.0 $CaCl_2$, 1.17 $MgSO_4$, 2.0 MOPS, 1.2 NaH_2PO_4, 5.0 glucose, 2.0 pyruvate, 0.02 EDTA, 2.75 NaOH (the pH of the solution was adjusted to 7.40 ± 0.02 at 37°C). A third order branch of the superior mesenteric artery was then carefully dissected free of adherent tissue. A segment of mesenteric artery (internal diameter circa 200 - 300 μm) was cut and then cannulated at each end with a glass pipette (external diameter 150 μm) and positioned in a 10 ml temperature-regulated chamber (Confocal Pressure Myograph System, 120CP, Danish Myo Technology). To avoid luminal flow, the upstream and downstream pressure remained equal throughout the experiment. After equilibration at 37°C for 20 mins, arteries were longitudinally stretched with a micrometer during maximal inflation (at 80 mmHg), and then maintained at 50 mmHg for the remainder of experiments. This protocol was found to optimize the vasomotor responses to agonists. The pressurized artery was visualised using a laser scanning confocal microscope (FV300- SU, Olympus) using a 10x objective (Olympus) and images were recorded with Fluoview software (Olympus) at 1 Hz. Artery outer diameter was recorded offline using edge-detection software (MetaMorph, Universal Instruments) (see figure 2.2 for summary). All arteries were constricted with 3 μM phenylephrine, and

concentration-response curves obtained following cumulative additions of ACh in a static bath at 37 °C.

2.2.2 Measurement of endothelial cell Ca^{2+}

In separate experiments, following equilibration of pressurized arteries, filtered (0.2 μm pore) MOPS-buffered solution containing the fluorescent Ca^{2+} indicator fluo-4 AM (25 μg/ml) and pluronic (0.05%) was perfused through the artery lumen for 10 minutes to enable the selective loading of the dye into endothelial cells (see figure 2.3). The composition of the fluorescent dye solution given was found to give a strong fluorescent signal and limit the entrance of dye into vascular smooth muscle. Fluorescence intensity was measured using a laser scanning confocal microscope (FV300-SU, Olympus, excitation 488 nm, emission 505 nm) using a 20x objective (water immersion, 3.3mm working distance, Olympus) and recorded with Fluoview software (Olympus) at 2 Hz. During acquisition, the plane of focus was lowered to the endothelial layer, and to enable continuous observation of individual endothelial cells, arteries were stimulated with ACh from resting diameter and not preconstricted. In some experiments, the voltage-gated Ca^{2+} channel blocker nifedipine (1 μM) was added to fully dilate arteries with myogenic tone, and to prevent contraction to 35 mM KCl. Cytochalasin-D, a cell permeable fungal toxin which binds actin filaments inhibiting both the association and dissociation of subunits, was also tested at various concentrations as a vessel immobiliser, whilst it effectively inhibited vessel movement it also caused some dye aggregation so was deemed an inferior methodology to the use of nifedipine. Since these VGCC are not found in endothelial cells, there was no effect of nifedipine on the endothelial Ca^{2+} response to ACh.

In experiments where Ca^{2+} was omitted from the MOPS-buffered solution, both the bath and luminal solutions were exchanged, and arteries were incubated for at least 10 minutes in Ca^{2+}-free buffer. In fluorescence studies, fluo-4 AM solutions were prepared using Ca^{2+} free buffer.

2.2.3 Solutions and drugs

Drugs were all from Sigma except for cyclopiazonic acid (CPA, Calbiochem), apamin (Latoxan) and 1-[(2-chlorophenyl) diphenylmethyl]-1*H*-pyrazole (TRAM-34, selective IK_{Ca} blocker, a generous gift from Dr H Wulff). CPA and TRAM-34 were dissolved in DMSO and then diluted in physiological buffer for experimentation. Control experiments indicated that the DMSO vehicle had no effect. All other stock solutions were prepared using distilled water. When used, inhibitors were added to the incubation solution and arteries or cells equilibrated for at least 15 min (apamin approximately 30 minutes) prior to obtaining responses. Fluo-4 AM and pluronic (F-127) were from Molecular Probes.

2.2.4 Data analysis

In diameter based experiments, results are summarized as means ± S.E.M. of *n* replicates, where n = number of vessels studied (each vessel form an individual animal). Average diameter values were expressed as a percentage dilatation of phenylephrine contracted arteries, where diameter following constriction taken as 0% dilalatation, and 100% dilatation being equivalent to the diameter of pressurized vessels in Ca^{2+} free buffer, or in the presence of 1μM

papaverine. Statistical comparisons were made using one way ANOVA with Bonferroni's post test. In fluorescence based experiments, average changes in $[Ca^{2+}]_i$ are summarized as means ± S.E.M. of *n* replicates, where n = 10 second averages of 16 individual cells from one vessel) were indicated by the fluorescence intensity (F) divided by the fluorescence intensity recorded immediately before application of agonist (F_0). Oscillation peaks were classified mathematically in order to objectively analyse oscillatory behaviour (16 individual cells per *n*). The peak of an oscillation, where a, b and c are consecutive data points, was defined as when b-a > 0, b-c > 0 and (b-a)+(b-c) > 1.4d, where d = maximum change in baseline fluorescence (noise).

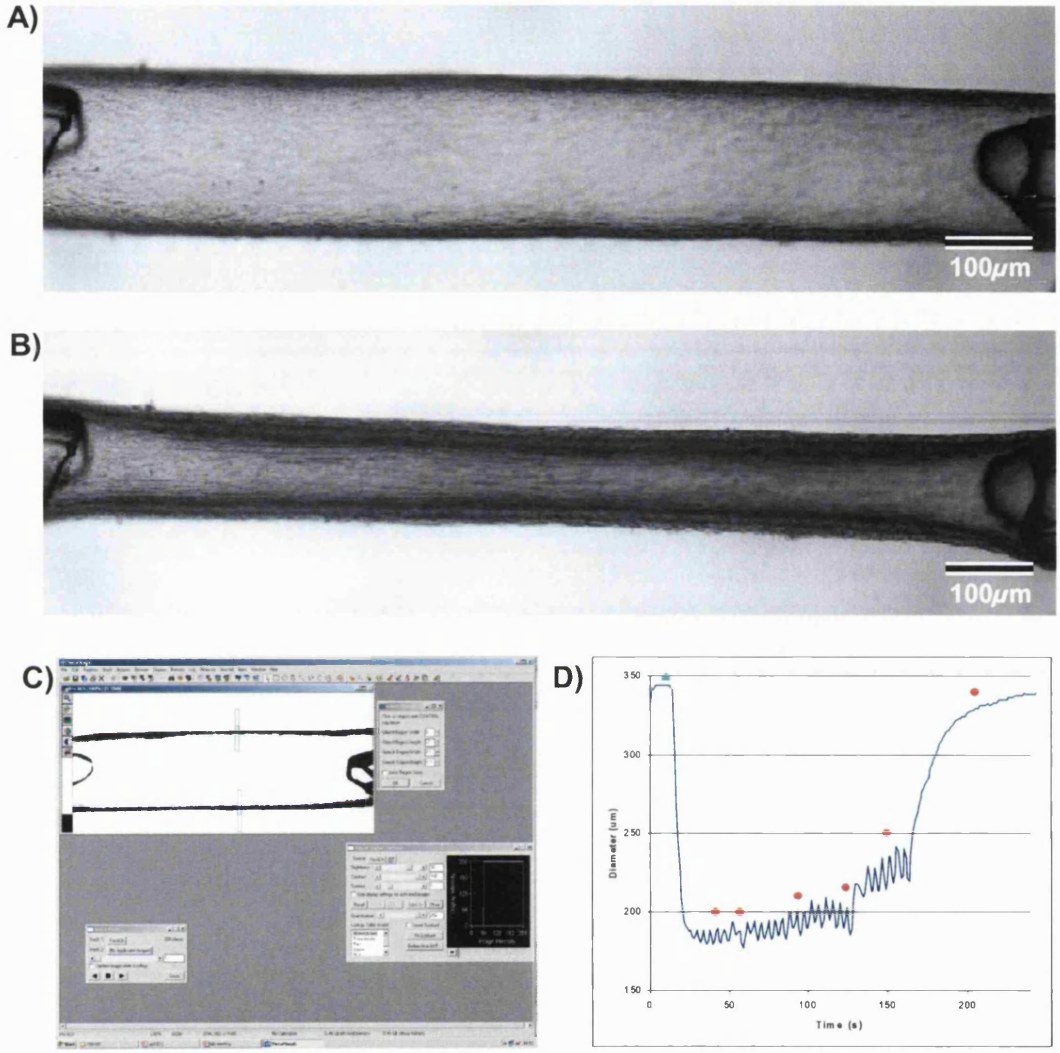

Figure 2.2; Measurement of vessel diameter using pressure myography. (A) and (B) Vessels are cannulated at either end and secured into position, scale bar = 100μm. (A) Following the elevation of intraluminal pressure, vessels inflate to their passive diameter. (B) Cannulated vessels respond to contractile agents with uniform constriction along the length of the vessel. (C) Diameter, internal or external as required, may then be calculated offline using edge detection software, producing a typical trace (D) detailing precise vessel movements.

2.3 MOUSE MESENTERIC STUDY

Male BALB-C mice (9-12 weeks old) were killed by cervical dislocation and exsanguination (Schedule 1 procedure; UK Animals (Scientific Procedures) Act 1986).

2.3.1 Pressure myography

The mesenteric arcade was removed and placed in chilled MOPS buffer (4° C) containing (mM): 145 NaCl, 4.7 KCl, 2.0 $CaCl_2$, 1.17 $MgSO_4$, 2.0 MOPS, 1.2 NaH_2PO_4, 5.0 glucose, 2.0 pyruvate, 0.02 EDTA, 2.75 NaOH (the pH of the solution was adjusted to 7.40 ± 0.02 at 37 °C). A second order branch of the superior mesenteric artery was then carefully dissected free of adherent tissue. A segment of mesenteric artery (internal diameter circa 100 - 250 μm) was cut and then cannulated at each end with a glass pipette (external diameter 100 μm) and positioned in a 10 ml temperature-regulated chamber (Confocal Pressure Myograph System, 120CP, Danish Myo Technology). To avoid luminal flow, the upstream and downstream pressure remained equal throughout the experiment. After equilibration at 37 °C for 20 mins, arteries were longitudinally stretched with a micrometer during maximal inflation (at 50 mmHg), and then maintained at 40 mmHg for the remainder of experiments. In pilot studies, this was found to be optimal for contraction to phenylephrine as assessed by exposure at various perfusions pressures (10–60mmHg). Previously a transmural pressure of 40mmHg (Boyle *et al.*, 2000) has been used sucessfully in isolated mouse mesenteric vessels. The pressurized artery was visualized using a laser scanning confocal microscope (FV300- SU, Olympus, Japan) using a 10x objective (Olympus) and images were recorded with Fluoview

software (Olympus, USA) at 1 Hz. Artery outer diameter was recorded offline using edge-detection software (MetaMorph, Universal Instruments) (for summary see figure 2.2). All arteries were constricted with phenylephrine, and concentration-response curves obtained following cumulative additions of SLIGRL in a static bath at 37 °C.

2.3.2 Immunohistochemistry

Following standard cannulation and testing of vessel viability, consisting of a sustained constriction to 3 μM phenylephrine in the presence of 100μM L-NAME and a >90% dilatation to 10 μM SLIGRL. Arteries were incubated in 2% paraformaldehyde for 10 min. The fixed arteries were washed with PBS, and permeabilized with a PBS solution containing Tween20 (0.1%) and BSA (0.1%, Sigma, A3059) for 1 hour at 37°C. The primary antibody (1:100 in permeabilization buffer) was perfused through the lumen and added to the bath to ensure reliable exposure to both endothelial and smooth muscle cells. Primary antibodies were: rabbit anti-human SK3 (Alomone, APC-025, 100% homology to mouse sequence), rabbit anti-rat SK4 (IK1; Alomone, APC-064, 100% homology to mouse sequence), rabbit anti-mouse BK (α-subunit, Alomone, APC-021, 100% homology to rat), and mouse anti-mouse α-smooth muscle actin (Sigma, A5228). Cannulated arteries were kept overnight at 4°C. Tissue was subsequently washed in PBS at room temperature, and endothelial and smooth muscle labelling determined independently. This was achieved by luminal perfusion of arteries with secondary antibody, rinsing with PBS after 30 mins, and acquiring images for endothelial cell-specific staining. The secondary antibody was then added to the bath for at least 1 hour, and images of the adventia, smooth muscle and endothelial cells were obtained with z-stacks

through the arterial wall. Secondary antibodies and their dilutions were: goat anti-rabbit Alexa 488 (1:100; Molecular Probes, A11034), goat anti-rabbit Alexa 633 (1:100; Molecular Probes, A21071) or goat anti-mouse Alexa 488 (1:200; Molecular Probes, A11029). Arteries were carefully transferred to a coverslip and mounted in buffered glycerol to obtain high-resolution images of flat segments (equivalent to *en face* imaging). All preparations were either stained with propidium iodide (5 μg/ml) to identify cell type or the autofluorescence of the internal elastic lamina was visualized to show sites of potential endothelial to smooth muscle cell contact (holes in the internal elastic lamina). All images were acquired using an Olympus FluoView500 confocal microscope using a 40x objective (water immersion 0.8NA, 3.3mm working distance) for pressurized arteries and a 60x objective (oil immersion 1.4NA, 0.1mm working distance) for flat segments. Laser and photomultiplier settings were matched between antibodies.

2.3.3 Solutions and drugs

Antibodies used for immunostaining were as described in the text, all other drugs were from Sigma except for apamin (Latoxan) and 1-[(2-chlorophenyl) diphenylmethyl]-1*H*-pyrazole (TRAM-34, selective IK_{Ca} blocker, a generous gift from Dr H Wulff). TRAM-34 was dissolved in DMSO and then diluted in physiological buffer for experimentation. Control experiments indicated that DMSO vehicle had no effect. All other stock solutions were prepared using distilled water. When used, inhibitors were added to the incubation solution and arteries or cells equilibrated for at least 15 min (apamin approximately 30 minutes) prior to obtaining responses. Fluo-4 AM and pluronic were from Molecular Probes.

2.3.4 Data analysis

Results are summarized as means ± S.E.M. of *n* replicates. Statistical comparisons were made using one way ANOVA with Bonferroni's post test. Average diameter values were expressed as a percentage dilatation of phenylephrine contracted arteries, with diameter following constriction taken as 0% dilalatation, and 100% dilatation being equivalent to the diameter of pressurized vessels in Ca^{2+} free buffer.

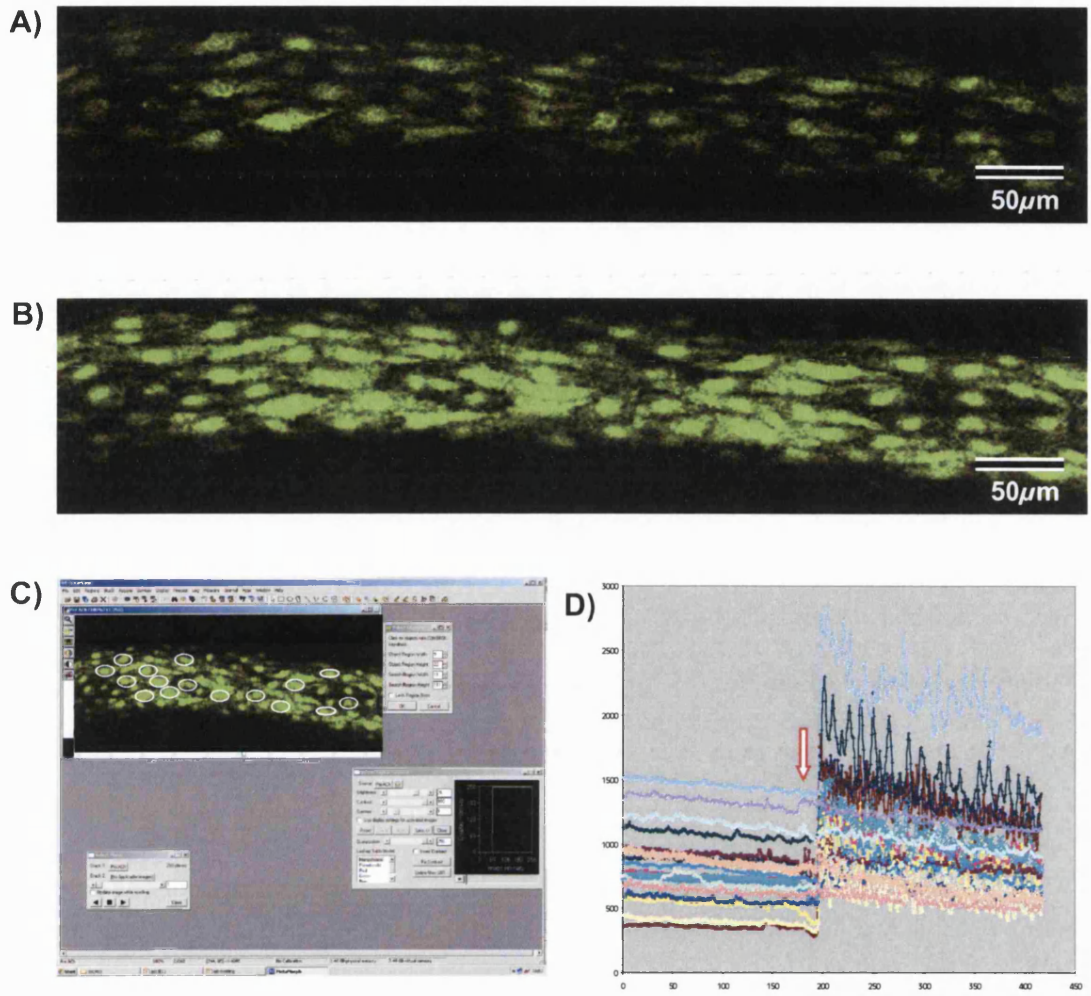

Figure 2.3; Measurement of endothelial cell Ca^{2+} using pressure myography.

(A) Loading of the Ca^{2+}-sensitive dye Fluo-4AM through the vessel lumen results in selective endothelial cell dye loading. (B) Activation of endothelial cell results in elevations in endothelial cell intracellular Ca^{2+} across the whole of the vessel section. (C) Changes in fluorescence of individual cells were monitored offline, producing a typical trace (D) detailing elevation and oscillation in the levels of endothelial cell Ca^{2+}, upon administration of 300nM ACh (arrow).

2.4 RAT CREMASTER STUDY

2.4.1 Tissue preparation

All experiments were performed using male Wistar rats (200-250g). Rats were anaesthetised with urethane ($2.8g.kg^{-1}$ i.p.) prior to excision of the cremaster muscle. Urethane has previously been shown not to alter the NO/EDHF balance (de Wit *et al.*, 1999) as other anaesthetics have been demonstrated to do (de Wit *et al.*, 1999; Loeb *et al.*, 1997). Following tissue removal rats were killed by cervical dislocation and exsanguination (Schedule 1 procedure; UK Animals (Scientific Procedures) Act, 1986). The cremaster muscle was placed in chilled MOPS buffer (4° C) containing (mM): 145 NaCl, 4.7 KCl, 2.0 $CaCl_2$, 1.17 $MgSO_4$, 2.0 MOPS, 1.2 NaH_2PO_4, 5.0 glucose, 2.0 pyruvate, 0.02 EDTA, 2.75 NaOH (pH 7.40 ± 0.02) and first order arteries were dissected free of surrounding and adherent tissues.

2.4.2 Isolated vessel pressure myography

A segment of secondary cremaster artery was cut and then cannulated at each end with a glass pipette (external diameter 75μm) and positioned in a 1.5ml temperature-regulated chamber. To avoid luminal flow, the upstream and downstream pressure remained equal throughout the experiment. Vessel segments were gradually pressurized to 75mmHg and warmed to 34°C (testicular temperature) during a 60 minute equilibration period. During this period, vessels were checked for pressure leaks and allowed to develop spontaneous basal tone. Vessel length was adjusted before the development of spontaneous tone by increasing segment length such

that at 75mmHg the vessel was stretched 100% of its passive length. The vessel preparation was positioned on the stage of an inverted microscope equipped with a video-based imaging system. Measurements of internal vessel diameter (in μm) were made using an electronic video calliper or edge-detection software (Diamtrack) (for summary see figure 2.2). Following equilibration, and development of tone, vessels showing a >90% relaxation to ACh were incubated with 100μM L-NAME and concentration-response curves were constructed to cumulative additions of ACh in the presence of various inhibitors.

2.4.3 Measurement of endothelial cell Ca^{2+}

A MOPS-buffered solution containing the fluorescent Ca^{2+} indicator fluo-4 AM (2.5 μg/ml) and pluronic (0.005%) was perfused through the artery lumen for 10 minutes to enable the selective loading of the dye into endothelial cells. The composition of the fluorescent dye solution given was found to give a strong fluorescent signal and limit the entrance of dye into vascular smooth muscle. Fluorescence intensity was measured using a laser scanning confocal microscope (FV300-SU, Olympus) and recorded with Fluoview software (Olympus) at 2 Hz (for summary see figure 2.3). During acquisition, vessels were pressursied to 75mmHg and the plane of focus was lowered to the endothelial layer. To enable continuous observation of individual endothelial cells, arteries were stimulated with ACh from resting diameter in the presence of the voltage-gated Ca^{2+} channel blocker nifedipine (1 μM) was added to fully dilate arteries against myogenic tone.

2.4.4 Electron microscopy

Rats anaesthetized as above were perfusion fixed using modified standard procedures (Sandow et al. 2002, *Circ Res*). In brief, animals were perfused via the left ventricle with a clearing solution of 0.1% BSA or NDS, 30 nM NONOate or 0.1% $NaNO_3$ and 10 U/ml heparin and fixed with 1% paraformaldehyde, 3% glutaraldehyde in 0.1 M sodium cacodylate, with 35 mM betaine (Sigma, B2629), pH 7.4. Short segments of the cremaster, as above, were dissected free from the fixed tissue and processed for electron microscopy as previously described (Sandow *et al.*, 2002).

Transverse sections (~100 nm thick) were cut and photographed at x2,500 and MEGJs and their surrounding endothelial cell and smooth muscle cell regions photographed at x10,000 to x40,000 on a transmission electron microscope (performed by Dr Shaun Sandow, University of Bath).

2.4.5 Drugs used

1-[(2-chlorophenyl) diphenylmethyl]-1*H*-pyrazole (TRAM-34), a selective IK_{Ca} channel blocker (kind gift from Dr H. Wulff) was dissolved in DMSO and then diluted in physiological buffer for experimentation. *N*-methylsulfonyl-6-(2-propargyloxyphenyl)-hexanamide (MS-PPOH, synthesized and provided by Dr J. Falck) and 17-octadecynoic acid (17-ODYA) (Sigma), both CYP450 inhibitors, were dissolved in 100% ethanol prior to dilution for

experimentation. Similarly, 11-12-epoxyeicosatrienoic acid (11-12-EET) and 14-15-epoxyeicosatrienoic acid (14-15-EET) (both synthesized and provided by Dr J. Falck), both isomers which are produced through the action of CYP450-epoxygenase, were synthesised by Dr J. Falck and were diluted in 100% ethanol under an argon atmosphere and stored at -80°C prior to experimentation. Control experiments indicated that the vehicle treatment had no effect on vessel diameter. K_{Ca} channel inhibitors charybdotoxin, apamin, and iberiotoxin, were all obtained from Latoxan, all other drugs were obtained from Sigma. All other stock solutions were prepared using distilled water. When used, inhibitors were added to the incubation solution and arteries or cells equilibrated for at least 20 minutes (apamin for at least 30 minutes) prior to obtaining responses.

2.4.6 Data analysis

Results are summarized as means ± S.E.M. of *n* replicates. Diameter values are expressed as a percentage of the maximum dilatation of myogenically contracted arteries, with basal tone taken as 0% and diameter following Ca^{2+} free MOPS incubation as 100% dilated. Statistical comparisons were made using one way ANOVA with Bonferroni's post test.

Chapter Three

EDHF-mediated dilatations in rat isolated small mesenteric arteries and the control of endothelial Cell Ca^{2+}

3.1 INTRODUCTION

Activation of endothelial cells with ACh has been consistently characterised by increases in $[Ca^{2+}]_i$ and membrane potential hyperpolarization, and is linked to the production of vasodilatatory substances including NO and EDHF. ACh acts via muscarinic type-3 (M_3) receptors (Fujimoto *et al.*, 1991), which are coupled to $G_{q/11}$ (Blin *et al.*, 1995), activation of which increases the formation of IP_3 and DAG and leads to sustained increases in $[Ca^{2+}]_i$ in a concentration-dependent manner (Nash *et al.*, 2001). The Ca^{2+} response can be split into two distinct components, an initial response which is dependent on IP_3 mediated release of Ca^{2+} from intracellular stores, and the sustained phase which is dependent on the entry of extracellular Ca^{2+} (Fukao *et al.*, 1997).

The earliest studies of endothelial cell Ca^{2+} were performed in isolated or cultured endothelial cells. The results of these numerous studies demonstrated that endothelial cell activation was associated with membrane potential hyperpolarization, and it was hypothesized that this hyperpolarization upon activation likely caused Ca^{2+} entry by increasing the electrical gradient for Ca^{2+} entry (Luckhoff *et al.*, 1990), as, contrary to vascular smooth muscle cells, voltage-gated Ca^{2+} channels were found to be of nominal importance in endothelial cells (Cannell *et al.*, 1989; Colden-Stanfield *et al.*, 1987). Conversely endothelial cell membrane depolarization has been demonstrated to inhibit Ca^{2+} entry in isolated cells (Wang *et al.*, 1999). Oscillations in endothelial cell $[Ca^{2+}]$i have been observed in cultured endothelial cell preparations grown to confluencey, oscillations were sychronized in response to bradykinin in pulmonary artery endothelial cells (Sage *et al.*, 1989) and bovine atrial valve endothelial cells (Laskey *et al.*,

1992). Oscillations in endothelial cell membrane potential that closely match Ca^{2+} oscillations have also been described in cultured cell preparations (Laskey *et al.*, 1992).

Arguably a more physiological approach than the study of isolated or cultured cells is the study of endothelial cell Ca^{2+} in whole vessels (Fukao *et al.*, 1997; Knot *et al.*, 1999; Ghisdal *et al.*, 2001; Ungvari *et al.*, 2001; Marrelli *et al.*, 2003; Cohen *et al.*, 2005), as homocellular electrical coupling exists between endothelial cells and between smooth muscle cells through gap-junctions. In addition, in some vessels, including rat mesenteric arteries, heterocellular electrical coupling occurs between endothelial and smooth muscle cells via myoendothelial gap junctions (Sandow *et al.*, 2002; White *et al.*, 2000). Whole vessel studies (Cohen *et al.*, 2005; Knot *et al.*, 1999; Marrelli *et al.*, 2003; McSherry *et al.*, 2005a; McSherry *et al.*, 2005b; Ungvari *et al.*, 2001) agree that activation of endothelial cells results in sustained endothelial cell Ca^{2+} increases, which has been demonstrated to be dependent on extracellular Ca^{2+} (Fukao *et al.*, 1997). Also, in rat mesenteric artery endothelial cells, the rise in $[Ca^{2+}]_i$ has been shown to activate K^+ channels (Walker *et al.*, 2001) resulting in K^+ efflux. Furthermore, Ca^{2+} handling may be affected by transmural pressure, as transmural pressure has been demonstrated to affect resting membrane potential of cannulated arteries (Harder, 1984), offering further physiological significance to pressurized whole vessel methodology. The studies utilising pressurized vessels that examined Ca^{2+} entry mechanisms (Knot *et al.*, 1999; Marrelli *et al.*, 2003; McSherry *et al.*, 2005a) produce contradictory evidence regarding the role of membrane potential in endothelial cell Ca^{2+} entry, with some studies suggesting a minimal effect of membrane potential (Marrelli *et al.*, 2003; McSherry *et al.*, 2005a), whereas others demonstrate decreased Ca^{2+} entry into depolarized endothelial cells (Knot *et al.*, 1999). Furthermore, in a

non-pressurized whole vessel study using rat superior mesenteric arteries, (Ghisdal *et al.*, 2001) report that blocking endothelial cell hyperpolarization to ACh, through blockade of SK_{Ca} and IK_{Ca} channels, did not affect endothelial cell Ca^{2+}, whereas elevating extracellular K^+ to depolarize endothelial cells inhibits endothelial cell Ca^{2+} entry producing a mixed view on the relevance of endothelial cell membrane potential on Ca^{2+} entry in these cells.

The routes and causes of extracellular Ca^{2+} entry during sustained responses in intact pressurized arteries remain to be fully elucidated, as such this study was designed to test two main hypotheses for the entrance of extracellular Ca^{2+} into agonist activated endothelial cells; i) membrane potential hyperpolarization leads to increases in the electrical gradient for Ca^{2+} entry, or ii) intracellular Ca^{2+} store depletion or second messengers facilitate Ca^{2+} entry. Furthermore the nature of the endothelial cell $[Ca^{2+}]_i$ elevations and oscillations observed was investigated in order to unravel the underlying mechanisms generating these profiles, and to test hypotheses regarding the control of membrane potential over endothelial cell Ca^{2+} dynamics formulated from the use of cultured and freshly isolated cells.

3.2 METHODS

Experiments were performed according to section 2.2

3.3 RESULTS

3.3.1 Effect of ACh on endothelial cell $[Ca^{2+}]_i$

Luminal perfusion of fluo-4 AM through cannulated rat mesenteric arteries selectively loaded endothelial cells (figure 3.1). In the absence of agonist stimulation, endothelial cells tended to remain quiescent. Application of 0.3μM ACh stimulated increases in endothelial cell $[Ca^{2+}]_i$ that were maintained over the period of stimulation (F/F_0 = 1.57 ± 0.03 at 10 s, F/F_0 = 1.30 ± 0.03 at 100 s, n = 10). Following stimulation, Ca^{2+} waves were observed to pass along the longitudinal axis of most cells. Generally, the waves appeared to originate at the longitudinal extremities of the cells and travelled unidirectionally (figures 3.2 & 3.3). However, more than one wave could occur within a single cell, resulting in superimposed waves that appeared to travel bidirectionally. The period and amplitude of oscillations within a single cell were irregular, possibly reflecting such superimposed Ca^{2+} waves. The $[Ca^{2+}]_i$ oscillations did not appear to synchronize between cells, either with adjacent cells or the cells across the entire field of view (figure 3.4).

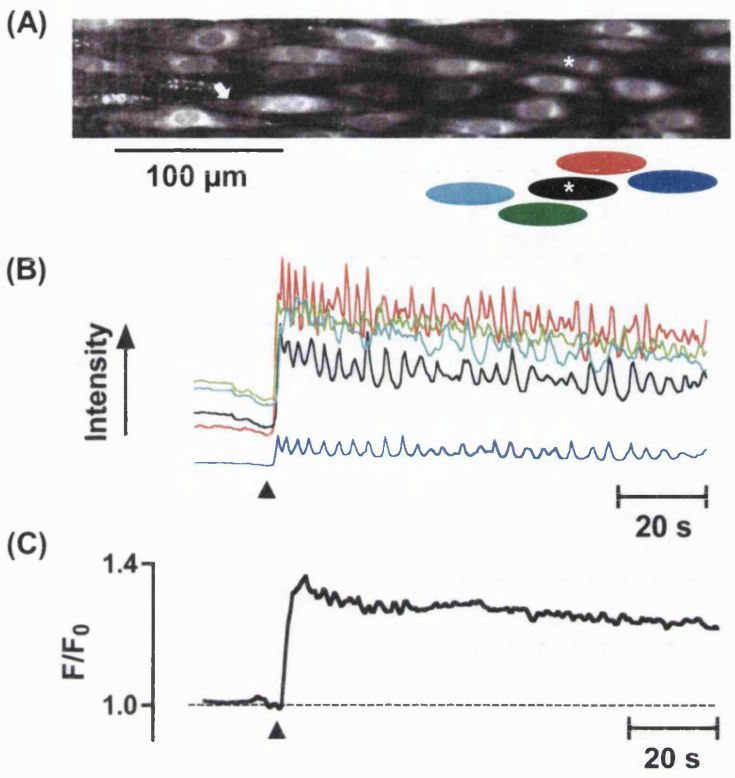

Figure 3.1; Effect of ACh on endothelial cell $[Ca^{2+}]_i$ in pressurized arteries. (A) Confocal fluorescence image of arterial endothelial cells loaded with fluo-4. (B,C) The time course of endothelial cell fluorescence changes in response to 0.3 µM ACh added at the time indicated (▲). (B) The average whole-cellular fluorescence intensity over time. The coloured lines correspond to the individual cells marked in (A), note that the oscillations in fluorescence were asynchronous. (C) The average multi-cellular relative fluorescence (F/F0) over time. The region of interest for measuring fluorescence intensity was increased to include all the cells in the field of view (> 50 cells). Note that the pattern of oscillations was markedly dampened compared to the individual cells, reflecting the asynchronous signals.

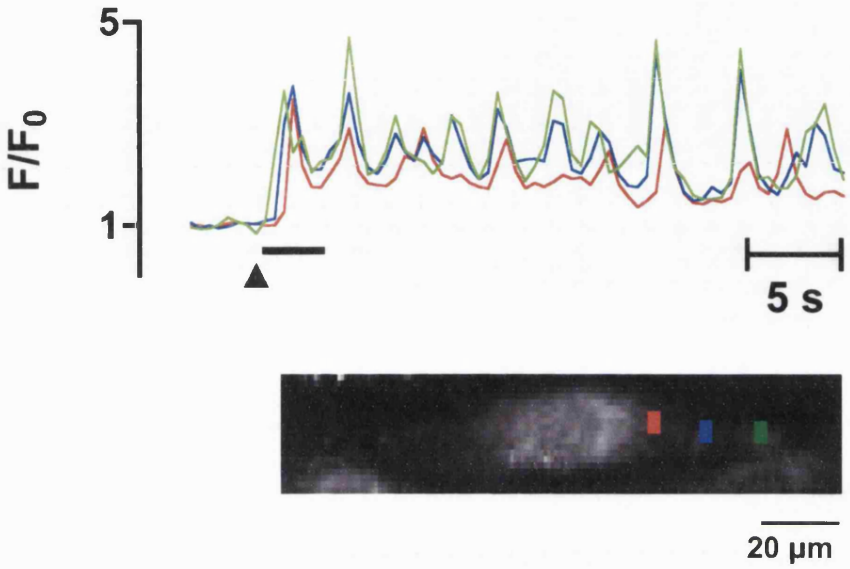

Figure 3.2; Effect of ACh on endothelial cell $[Ca^{2+}]_i$ in pressurized arteries. Changes in F/F0 in the regions corresponding to the coloured boxes indicated over the cell below. Note that the increases in F/F0 started moving from right to left, and were not uniform in amplitude, direction, or duration.

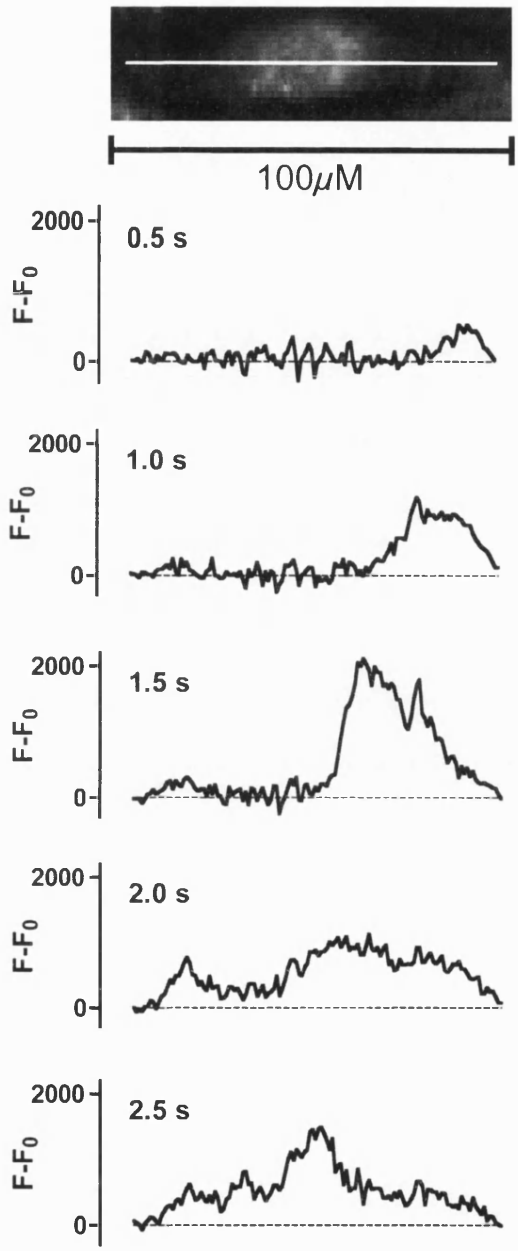

Figure 3.3; Effect of ACh on endothelial cell $[Ca^{2+}]_i$ in pressurized arteries. The difference in fluorescence intensity ($F-F_0$) across the length of the cell (white line in image) over time (a single oscillation indicated by the bar in the top panel). The dotted line in each panel corresponds to the *x*-coordinate in the image above (white line), and $t = 0$ s. The wave travelled from right to left at a speed of approximately 30 μm/s, and appeared to quench at the position of the nucleus.

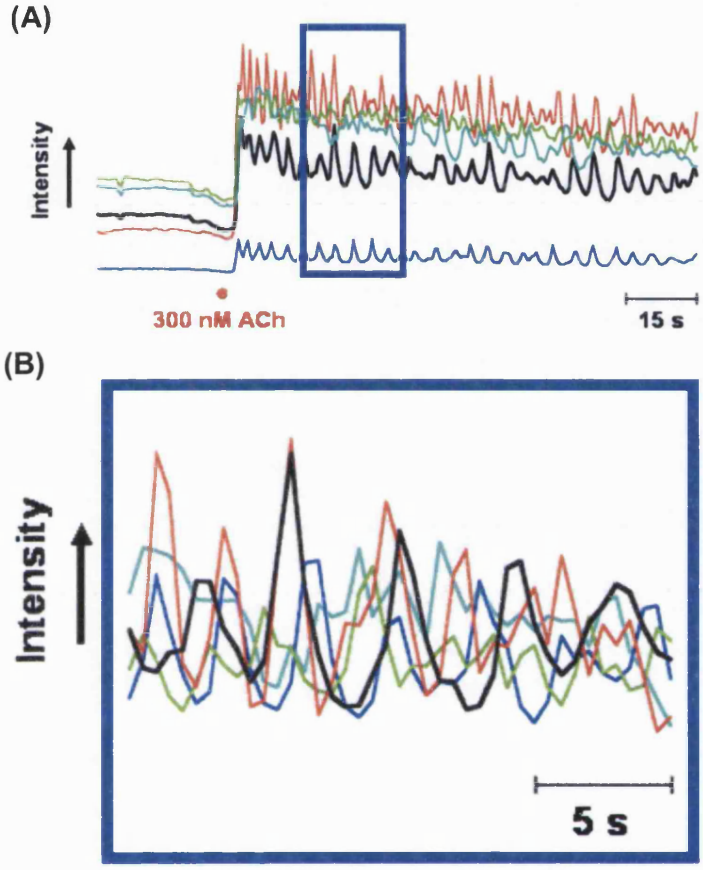

Figure 3.4; Asynchronous nature of endothelial cell Ca^{2+} oscillations following ACh application. (A) Each line in the figure represents the Ca^{2+} oscillations in endothelial cells, from the same vessel, following application of 300nM ACh (zoom window and overlay in (B)). Oscillations are unsynchronised between endothelial cells and exhibit differing frequencies and amplitudes of oscillation.

3.3.2 Role of membrane potential in driving Ca^{2+} influx

Application of ACh, in the presence of phenylephrine tone, caused concentration-dependent dilatation of rat mesenteric arteries (figures 3.5 & 3.6). The inhibition of ACh-induced L-NAME-insensitive dilatation of pressurized arteries required the presence of inhibitors of both SK_{Ca} and IK_{Ca}, with 50nM apamin and 1 μM TRAM-34 respectively (n=6, $p<0.05$), neither inhibitors alone (apamin, n=3; TRAM-34, n=3) were sufficient to fully inhibit ACh induced dilatation (figure 3.6). The profile of rises in endothelial cell $[Ca^{2+}]_i$ in response to 300nM ACh (n=10) was unaffected throughout the entire time course by SK_{Ca} and IK_{Ca} blockade (n=5, $p>0.05$) (figure 3.6). Given that joint SK_{Ca} and IK_{Ca} inhibition is known to inhibit endothelial cell hyperpolarization (Hinton *et al.*, 2003), the role of membrane potential was investigated further by preventing endothelial cell hyperpolarization by raising extracellular K^+ to 35 mM, this treatment was also without effect on endothelial cell Ca^{2+} responses (n=3, $p>0.05$).

3.3.3 Sources of Ca^{2+} in response to ACh

There were two clear phases to the time course of rises in endothelial cell $[Ca^{2+}]_i$ in response to 300nM ACh (figure 3.7). Removal of extracellular Ca^{2+}, had no effect on the initial rise in endothelial cell $[Ca^{2+}]_i$, but strongly attenuated the sustained increases (100s following ACh administration $F/F_0 = 0.93 \pm 0.04$, $n = 7$). Incubation of arteries with the inhibitor of the endoplasmic reticulum Ca^{2+}-ATPase, cyclopiazonic acid (CPA, 10μM, n=5) fully blocked endothelial cell $[Ca^{2+}]_i$ oscillations and inhibited Ca^{2+} rises during both the early (t = 0-30s) and late (t = 30-100s) phases of stimulation in response to 0.3μM ACh, whilst small increases

in endothelial cell Ca^{2+} were observed in the sustained phase in the presence of CPA. 300nM ACh was also demonstrated to elicit a sub-maximal endothelial cell Ca^{2+} response, by the increased Ca^{2+} response to 3μM ACh (n=3) (figure 3.7).

3.3.4 Affect of treatments on the percentage of oscillating cells and the oscillation frequency

The nature of endothelial cell Ca^{2+} oscillations was investigated further by examining the percentage of responding cells and the oscillation frequency in the presence of the various inhibitors utilised in this study (see figures 3.8 & 3.9). The average change in $[Ca^{2+}]_i$ within a single cell oscillated at an initial (t = 0-30s) frequency of 0.34 ± 0.03Hz (n = 10), and was approximately halved by lowering the chamber temperature to 22°C (0.18 ± 0.01Hz, n = 3). TRAM-34 and apamin (n=5) and 35mM extracellular K^+ (n=3) treatments did not affect either of these variables (both $p>0.05$). In experiments where the extracellular Ca^{2+} was removed (n=7) oscillations persisted in initial but not sustained phases. Following treatment with CPA (n=5) almost no oscillations were observed.

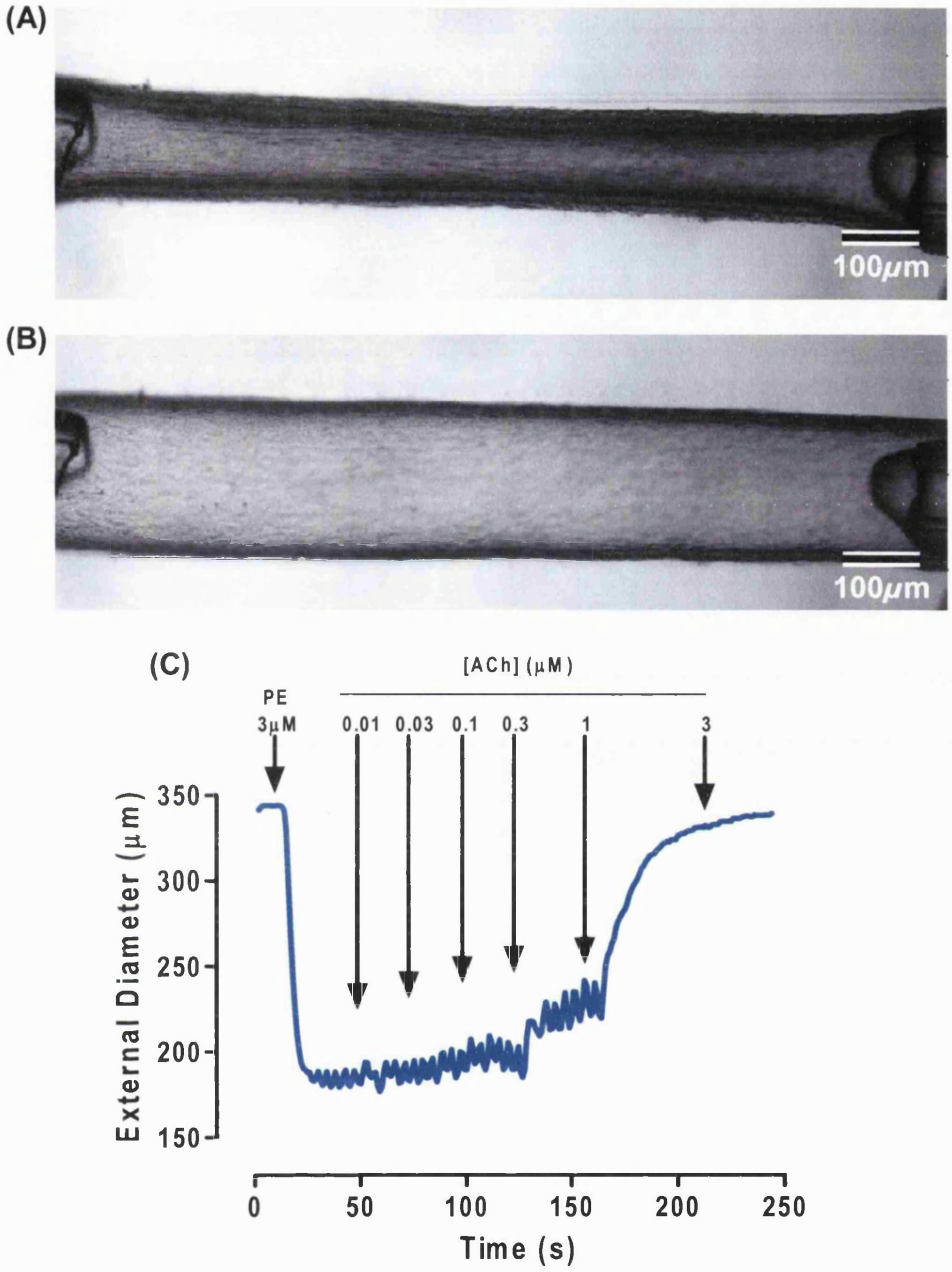

Figure 3.5; Dilatation of pressurized rat mesenteric arteries by ACh. Pressurized rat mesenteric arteries preconstricted with phenylephrine (A) dilatate on application of ACh (B). (C) Typical trace plotting the vessel external diameter following cumulative additions of ACh as indicated.

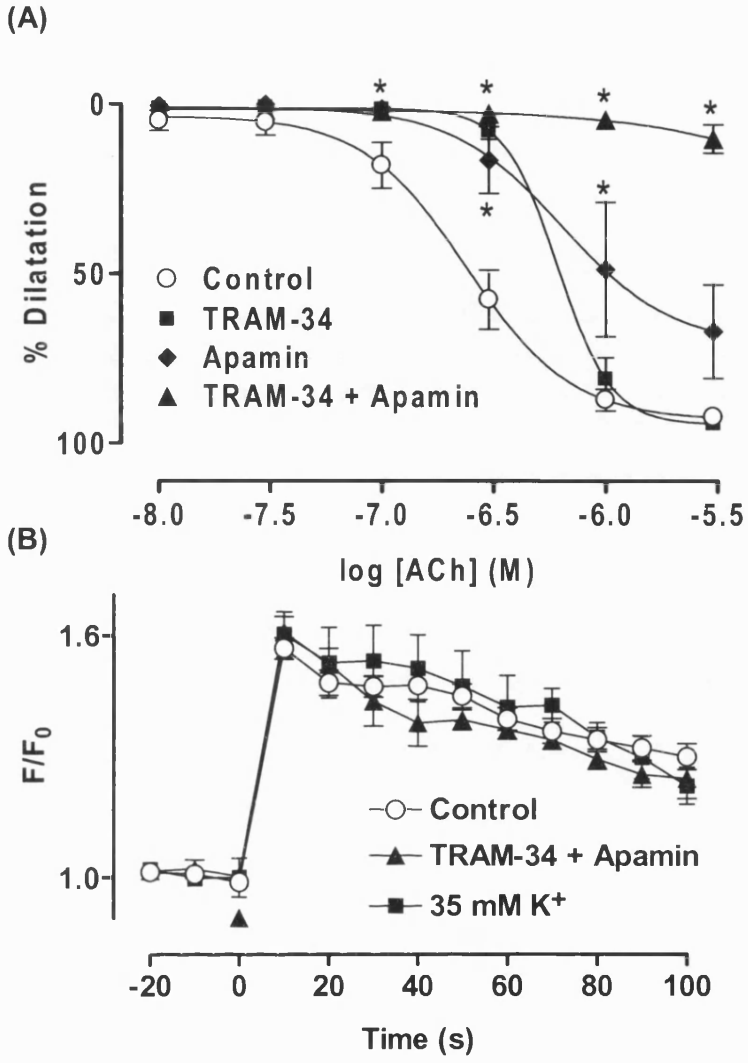

Figure 3.6; The role of SK_{Ca} and IK_{Ca} in EDHF responses and the control of endothelial cell Ca^{2+}. (A) In the presence of 100μM L-NAME (n=6) full dilatation to ACh may still be achieved. Blockade of either SK_{Ca} (50nM apamin, n=3), or IK_{Ca} (1μM TRAM-34, n=3) alone caused some attenuation of dilatation but was not sufficient to fully inhibit responses to ACh. The combined blockade of SK_{Ca} and IK_{Ca} was sufficient to block responses to 3μM ACh (n=6, $p<0.05$). (B) No difference in the profile of Ca^{2+} elevations following 300nM ACh administration were observed in the presence of either TRAM-34 and apamin (n=5) or 35mM K^+ (n=3). * denotes $p<0.05$ vs control.

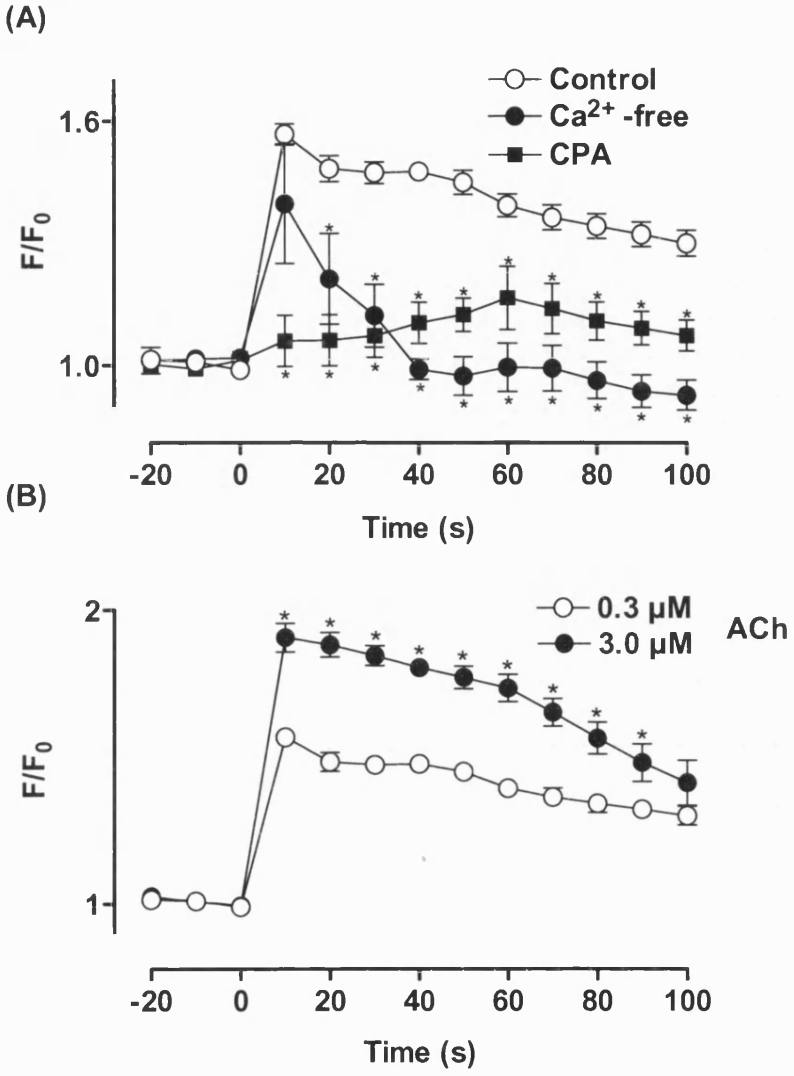

Figure 3.7; Sources of Ca^{2+} following ACh administration. (A) Following removal of extracellular Ca^{2+} (n=7), transient endothelial cell Ca^{2+} responses to ACh administration remained, sustained elevations in endothelial cell Ca^{2+} were ablated. CPA (n=5) inhibited transient Ca^{2+} responses, but did result in a slowly increasing endothelial cell Ca^{2+} in the sustained phase. (B) 300nM ACh elicits sub-maximal endothelial cell Ca^{2+} responses, as demonstrated by the larger increase in fluo-4 fluorescence following 3μM administration (n=3). * denotes $p<0.05$ vs control.

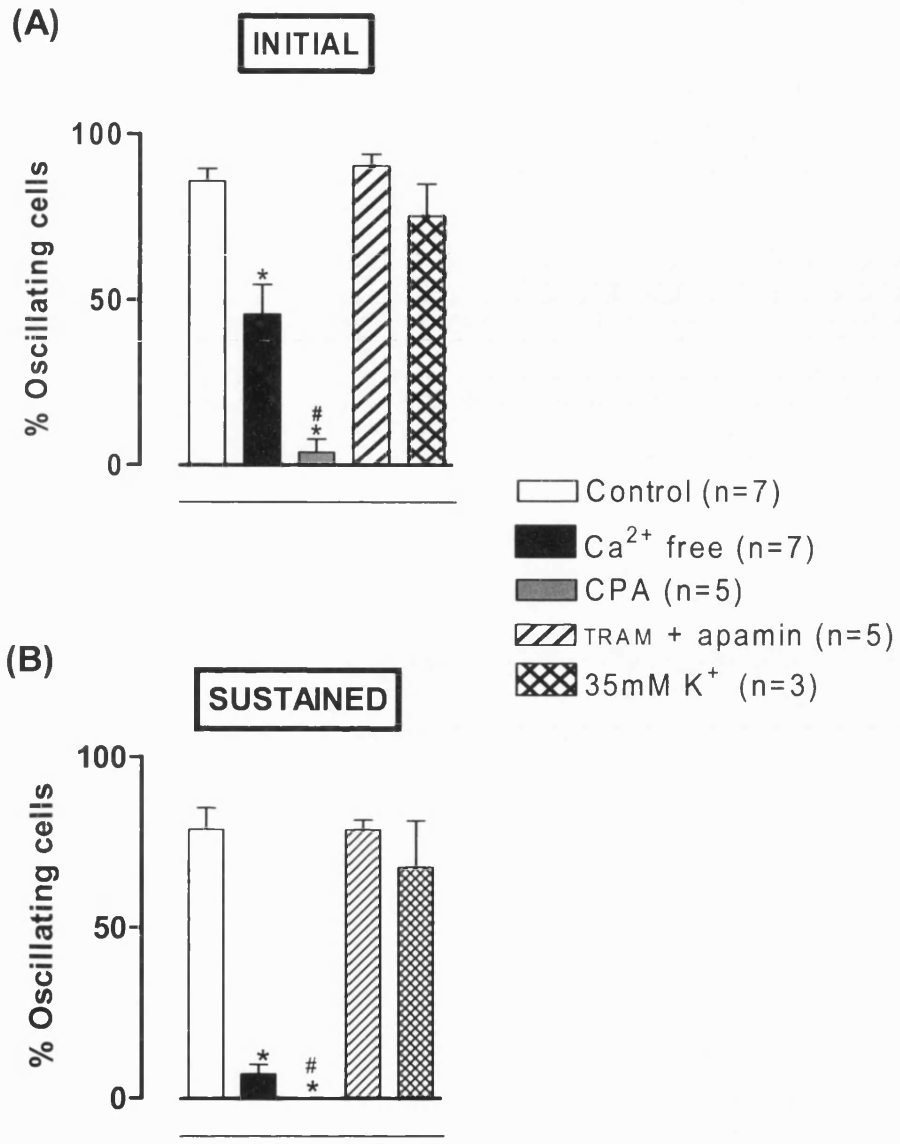

Figure 3.8; Effect of treatments on oscillations in endothelial cell $[Ca^{2+}]_i$ in pressurized arteries. The effect of the various treatments on the oscillations in endothelial cell $[Ca^{2+}]_i$ in response to ACh. The time course following the addition of 0.3 μM ACh to the bath was divided into two phases, (A) initial: t = 0-30 s; and (B) sustained: t = 30-100 s. The percentage of cells oscillating was calculated from the 16 cells analyzed per artery. * denotes $p<0.05$ vs control, # denotes $p<0.05$ vs Ca^{2+} free.

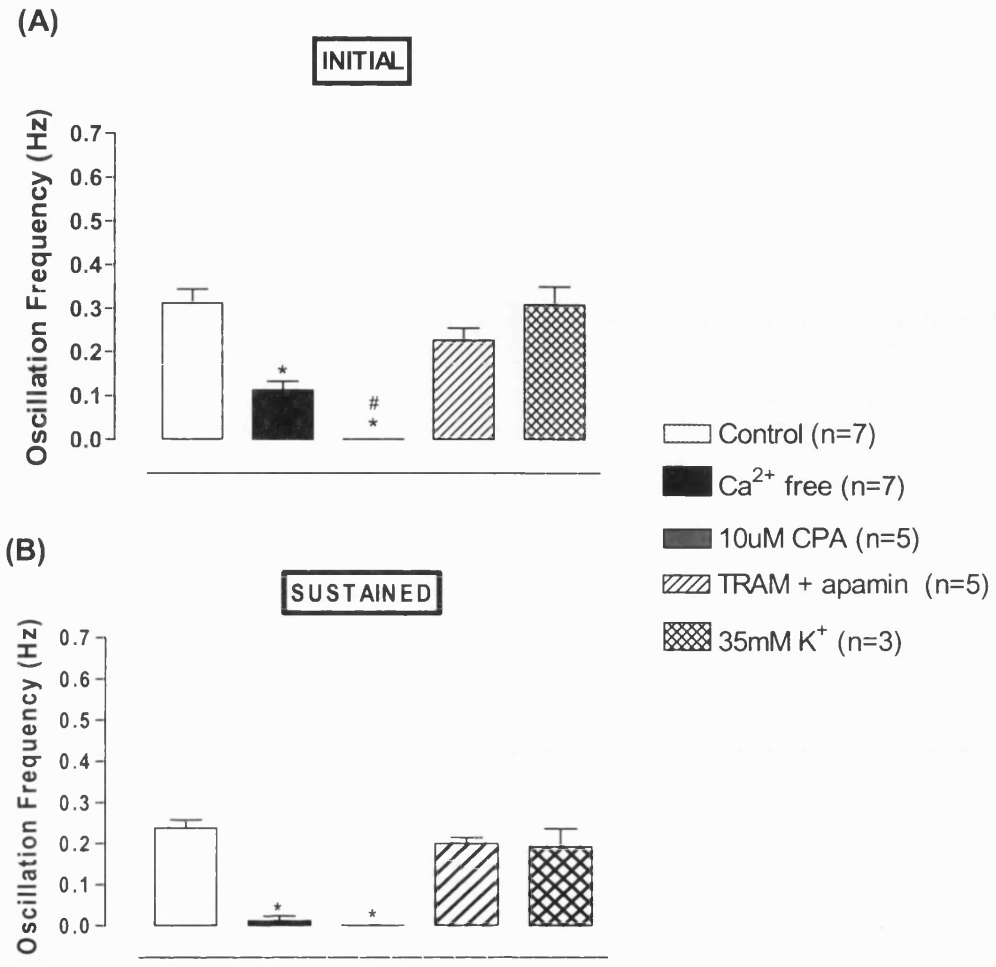

Figure 3.9; Effect of treatments on the frequency of oscillations in endothelial cell $[Ca^{2+}]_i$ in pressurized arteries. The effect of the various treatments on the frequency of oscillations in endothelial cell $[Ca^{2+}]_i$ in response to ACh. The time course following the addition of 0.3 μM ACh to the bath was divided into two phases, initial: t = 0-30 s; and sustained: t = 30-100 s. The frequency of oscillations were calculated analysing only oscillating cells. * denotes $p<0.05$ vs control, # denotes $p<0.05$ vs Ca^{2+} free.

3.4 DISCUSSION

3.4.1 Agonist induced increases in endothelial cell Ca^{2+}

Throughout this investigation ACh stimulated sustained elevations of pressurized rat mesenteric artery endothelial cells Ca^{2+} over a 100s time-course, as has been demonstrated previously in this artery (Takano *et al.*, 2004). Since M_3 receptors are confined to the endothelium in this vessel, the rises in $[Ca^{2+}]_i$ are entirely endothelium-dependent (Ghisdal *et al.*, 2001; Oishi *et al.*, 2001). M_3 agonists increase the concentration of IP_3 and DAG and lead to sustained increases in $[Ca^{2+}]_i$ in a concentration-dependent manner (Nash *et al.*, 2001).

Application of ACh to cell preparations where extracellular Ca^{2+} is removed results in an initial transient peak in $[Ca^{2+}]_i$, which is attributable to the formation of IP_3 and the release of Ca^{2+} from intracellular stores, with sustained elevations in endothelial cell $[Ca^{2+}]_i$ strongly attenuated (Carter *et al.*, 1994; Felder *et al.*, 1992; Luckhoff *et al.*, 1988; Sage *et al.*, 1989). However, there are no previous reports assessing this separation of Ca^{2+} signal in rat mesenteric artery endothelial cells. The data presented here demonstrates directly that the two sources of Ca^{2+} are also reflected in the Ca^{2+} increase to ACh in rat mesenteric artery endothelial cells. The removal of extracellular Ca^{2+} resulted in a transient elevation in endothelial cell $[Ca^{2+}]_i$ in response to ACh, whereas depletion of intracellular stores with CPA (10 μM) almost fully blocked the early phase (t = 0-30 s) of the Ca^{2+} response. This is consistent with both the M_3-mediated formation of IP_3 causing release of Ca^{2+} from intracellular stores, and activation of a Ca^{2+} influx pathway. In the absence of a selective and

cell-permeant inhibitor of IP_3 receptors, this could not be investigated further. However, in separate studies from our laboratory we have utilized the ability of an osmotic shock protocol to load heparin and anti-IP_3 receptor antibodies selectively into the endothelium of rat mesenteric arteries the results of which mimic and support the current findings (Mather *et al.*, 2005).

In the presence of CPA, ACh was still able to evoke a slowly developing rise in endothelial cell $[Ca^{2+}]_i$, which may reflect store mediated Ca^{2+} entry, a stimulation of the rate of Ca^{2+} influx from the extracellular solution following intracellular store depletion. The molecular nature of store medited Ca^{2+} entry pathways, and activation mechanisms remain elusive (for review see Nilius, 2003). A candidate channel which may be activated by store depletion is the TRP channel superfamily. However, TRP activation mechanisms have not been fully elucidated and likely vary between isoforms, and no selective inhibitors for the various isoforms exist, hence the involvement of TRP channels in this process remains speculative.

3.4.2 Role of membrane potential in driving Ca^{2+} influx

ACh stimulates robust endothelial cell hyperpolarization in rat mesenteric arteries from a resting membrane potential of approximately –55mV (White *et al.*, 2000; Tare *et al.*, 2002; Hinton *et al.*, 2003; Takano *et al.*, 2004) including in pressurized arteries (McSherry *et al.*, 2005b). The hyperpolarization to higher concentrations of ACh (>0.1μM) is maintained for the period of stimulation, does not show oscillatory patterns, and is sensitive to inhibitors of SK_{Ca} and IK_{Ca} channels (White *et al.*, 2000; Hinton *et al.*, 2003; McSherry *et al.*, 2005b). In the

current investigation, under conditions where hyperpolarization of endothelial cells in response to ACh was prevented, either through K_{Ca} inhibition or raising the extracellular $[K^+]$ to 35mM, the profile of rises in endothelial cell $[Ca^{2+}]_i$ remained unaffected. If Ca^{2+} influx were entirely or largely dependent on the electrochemical gradient, then the profile would be expected to mimic that of removing extracellular Ca^{2+} or blockade of Ca^{2+} influx pathways. Since this was not the case, it is unlikely that influx is driven to any significant degree by the hyperpolarization (<25mV, based on a resting membrane potential of –55mV, McSherry *et al.*, 2005b). This finding is consistent with a previous study using rat mesenteric arteries in a pinned out preparation (Takano *et al.*, 2004), in addition, studies utilizing pressurized rat middle cerebral arteries show there is no effect in that vessel of membrane potential on endothelial cell Ca^{2+} entry (Marrelli *et al.*, 2003). However, in rat coronary arteries there appears to be decreased Ca^{2+} entry into depolarized endothelial cells (Knot *et al.*, 1999). It is not known why the voltage-sensitivity of Ca^{2+} influx might vary between tissues, but it may relate to the expression profile of the Ca^{2+} entry channels (e.g. TRPC channels).

Whilst not observed at any stage of this study, previously synchronization of endothelial cell Ca^{2+} oscillations has been reported in cultured cells (Sage *et al.*, 1989). Given the reported membrane potential sensitivity of Ca^{2+} entry into cultured endothelial cells, this would demonstrate the ability of the electrically coupled cells to synchronize oscillations in membrane potential. The electrically coupled endothelial cells in the whole vessel preparation can also theoretically become electrically synchronized – yet no synchronization of Ca^{2+} oscillations are observed, suggesting further that endothelial cell membrane potential does not drive Ca^{2+} entry. In addition, observed endothelial cell hyperpolarizations to ACh results in a

steady non-oscillatory hyperpolarization, which conflicts with the oscillatory nature of endothelial cell Ca^{2+} elevations.

3.4.3 Endothelial cell Ca^{2+} oscillations

Individually, endothelial cells demonstrated oscillatory behaviour throughout the observed time-course. The oscillations occurred out of phase with adjacent cells, and when all the cells in the field of view were averaged (>50 cells), the pattern of rises in $[Ca^{2+}]_i$ did not oscillate, as has been reported previously (Kasai *et al.*, 1997; Ohi *et al.*, 2001; Ungvari *et al.*, 2002). Thus, the signals for oscillatory behaviour likely originated within each cell. In addition, waves of Ca^{2+} were observed in each cell, often originating at the terminal ends of cells, as described in another study (Kasai *et al.*, 1997), and may relate to the frequent spontaneous discharge sites observed in intact preparations (Burdyga *et al.*, 2003; Duza *et al.*, 2003). These sites may relate to the distribution of M_3 receptors, the accumulation sites for IP_3 and/or other signalling molecules and may also be caveolin-rich domains as described in cultured cells (Isshiki *et al.*, 1998). The majority of waves appeared to propagate unidirectionally, at a rate of approximately 30 µm/s. This was not investigated further due to the slow image acquisition rate, but falls into the range reported in other endothelial cell preparations (Burdyga *et al.*, 2003; Missiaen *et al.*, 1996) and intestinal smooth muscle cells (McCarron *et al.*, 2004). Out of phase bidirectional waves were also observed, which may reflect superimposed waves originating from different ends of the cell, or be a result of intercellular Ca^{2+} signalling between adjacent endothelial cells. Intercellular signalling may be attributable to the diffusion of a signalling molecule (e.g. IP_3, Carter *et al.*, 1996) through the high density of gap junctions

that have been shown to occur around the periphery of endothelial cells in this artery (Kansui *et al.*, 2004), or to the diffusion of an extracellular signalling molecule (e.g. ATP, Moerenhout *et al.*, 2001) between cells. Thus the observed non-uniform pattern of oscillations within an individual cell likely reflects both the time between the trigger for waves from one part of the cell, and the presence of superimposed waves in other parts of the cell.

In the presence of CPA no oscillations in endothelial cell Ca^{2+} were observed following application of ACh, demonstrating the necessity of a functional intracellular store for the generation and maintainace of endothelial cell Ca^{2+} oscillations. Oscillations in endothelial cell $[Ca^{2+}]_i$ were observed in Ca^{2+}-free conditions, a situation where the formation of IP_3 is expected (at least transiently) whereas Ca^{2+} influx is not. So there was a correlation between the formation of IP_3 and oscillations in $[Ca^{2+}]_i$, making it likely that oscillations are at least partially dependent on the formation of IP_3. The spread of a Ca^{2+} wave may depend on Ca^{2+}-induced Ca^{2+} release activating, or facilitating activation, of adjoining IP_3 receptors and/or ryanodine receptors, as demonstrated in cultured endothelial cells (Domenighetti *et al.*, 1998; Graier *et al.*, 1998) and freshly isolated intestinal smooth muscle cells (McCarron *et al.*, 2004). Oscillations in endothelial cell $[Ca^{2+}]_i$ in response to stimulation of G-protein coupled receptors have been reported in cultured endothelial cells (Jacob *et al.*, 1988; Sage *et al.*, 1989; Laskey *et al.*, 1992; Paltauf-Doburzynska *et al.*, 2000), and in intact arteries (Carter *et al.*, 1994; Kasai *et al.*, 1997; Ohi *et al.*, 2001). Of the intact artery preparations, the oscillation frequency ranged from approximately 0.03Hz (Carter *et al.*, 1994) to 0.1-0.3Hz (Kasai *et al.*, 1997; Ohi *et al.*, 2001), ranges similar to that reported in this investigation. In the latter two studies, it was

reported that inter-endothelial cell Ca^{2+} signalling did occur, but only as discrete events, similar to the present study.

3.4.4 EDHF mediated relaxations

The smooth muscle hyperpolarization (from resting membrane potential) elicited by ACh is sensitive to apamin alone (Chen *et al.*, 1997; Crane *et al.*, 2003a; McSherry *et al.*, 2005b), whereas IK_{Ca} contribute to the repolarization phase of relaxation, observed in the presence of arterial tone (Chen *et al.*, 1997; Crane *et al.*, 2003a). In the present study, the combination of apamin and TRAM-34 fully blocked the L-NAME insensitive component of dilatation to ACh, confirming our findings using the same arteries under isometric tension where membrane potential was simultaneously recorded (Crane *et al.*, 2003a).

3.4.5 Implications for the control of resistance artery diameter by endothelial cells

In rat mesenteric arteries, the presence of homocellular and heterocellular gap junctions provide the potential for effective longitudinal and radial electrical coupling (Sandow *et al.*, 2002; Takano *et al.*, 2004; White *et al.*, 2000), but intercellular Ca^{2+} signalling was not observed beyond an additional cell length (Takano *et al.*, 2004). Since stimulation with G-protein receptor-coupled agonists such as ACh clearly results in both local and spreading hyperpolarization and dilatation responses (Takano *et al.*, 2004), an understanding of the signalling events leading to changes in endothelial cell $[Ca^{2+}]_i$ provides insight into these processes. Further to this, data presented here confirm that hyperpolarization of endothelial

cells does not drive Ca^{2+} influx in this artery (Ghisdal *et al.*, 2001; Takano *et al.*, 2004), and suggest that store-depletion drives Ca^{2+} entry. Thus although the endothelium can coordinate vasomotor responses along the length of arteries, Ca^{2+} influx into endothelial cells is not a major driving force for this process. Rather, discrete rises in endothelial cell $[Ca^{2+}]_i$ can activate K_{Ca}, which may be spatially arranged (Crane *et al.*, 2003a; Frieden *et al.*, 2002), and evoke the spread of hyperpolarization between cells of the arterial wall.

Chapter Four

SLIGRL-mediated dilatations in mouse isolated small mesenteric arteries, and expression patterns of K_{Ca} isoforms

4.1 INTRODUCTION

Previous study of the mouse mesenteric artery has revealed hyperpolarization dependent 'EDHF' responses, which are independent of NO or prostacyclin, following selective endothelial activation. A recent study demonstrated endothelium-dependent hyperpolarization of vascular smooth muscle which was reliant on the transfer of hyperpolarization from the endothelium directly to underlying muscle via myoendothelial gap junctions (Dora *et al.*, 2003), negating the need for a putative 'factor', whereas others have identified K^+ (McGuire *et al.*, 2004) and H_2O_2 as possible mediators of EDHF responses in mouse mesenteric arteries (Matoba *et al.*, 2000).

ACh has been consistently described to increase endothelial cell $[Ca^{2+}]_i$ via activation of M_3 receptors (Fujimoto *et al.*, 1991), elicit membrane potential hyperpolarization, and to relax arteries in an endothelium dependent manner. Although ACh has been used with limited success in mouse mesenteric arteries (Matoba *et al.*, 2000), it has been reported to have reduced activity in mouse mesenteric arteries compared to other vessels (Dora *et al.*, 2003), perhaps reflecting sparse or differential expression of muscarinic receptors in this vascular bed. An alternative to ACh as an endothelium activating ligand is the hexapeptide sequence Ser-Leu-Ile-Gly-Arg-Leu (SLIGRL) which activates protease-activated receptors (PARs) type 2 (PAR2). PARs represent a class of seven transmembrane domain G-protein coupled receptors which are activated by proteolyitic cleavage, the mechanism for activation involving an unmasking of an N-terminal peptide sequence that acts as a tethered ligand, the activating sequence for murine PAR2 being SLIGRL (Nystedt *et al.*, 1994). PAR2 are extensively

expressed throughout the vasculature (Cicala, 2002; D'Andrea *et al.*, 1998), and a functional role for PAR2 on the vascular endothelium was first demonstrated through the endothelium dependent relaxation of rat thoracic aorta (al-Ani *et al.*, 1995). Subsequently, activation of PAR2 has been linked to the production of NO (Moffatt *et al.*, 1998; Sobey *et al.*, 1998) and EDHF (Kawabata *et al.*, 2003; McGuire *et al.*, 2002) from the endothelium. However, direct activation of PAR2 on vascular smooth muscle results in contraction (Moffatt *et al.*, 1998), thus the expression patterns of PAR2 are critical when considering the response to PAR2 agonists.

Agents generating arterial constriction may affect the dilatatory responses following endothelial cell activation according to their mode of action, as such the precise mechanisms underlying vessel constriction are important experimental considerations. Many contractile agonists have been proposed to stimulate the hydrolysis of phosphatidylinositol, via the action of phospholipase-C, to IP_3 and DAG. IP_3 causes release of Ca^{2+} from intracellular stores, whilst DAG is a putative activator of protein kinase-C, and may be directly involved in the activation of TRP channels and numerous intracellular signalling pathways, including modulation of the Ca^{2+} sensitivity of the contractile machinery, although its precise mechanisms of action are uncertain. The actions of DAG can be mimicked through the use of phorbol ester compounds, which have been demonstrated to activate protein kinase C through binding of the same activation domain as DAG (Blumberg *et al.*, 1984) and cause a sustained contraction in a number of blood vessels (Hattori *et al.*, 1995; Khalil *et al.*, 1988; Rasmussen *et al.*, 1984). Interestingly, the maximal contractile response to phorbol esters is increased in experimental models of diabetes (Abebe *et al.*, 1990). The current study investigates the mechanisms

through which DAG affects contraction and dilatation of arteries, in particular effects on the EDHF response, as changes in both PKC activity and EDHF responses have been reported in endothelial dysfunction associated with diabetes.

In this investigation the vasodilatory responses to SLIGRL were assessed in mouse mesenteric arteries and the generation of an EDHF-like response which was sensitive to the combined inhibition of IK_{Ca} and SK_{Ca} was observed. In light of this the cellular location of K_{Ca} isoforms were determined by immunohistochemistry, revealing a close correlation between holes in the internal elastic lamina and localised areas of high SK_{Ca} and IK_{Ca} expression. Phorbol ester resulted in sustained constriction of arteries, whilst no EDHF responses were observed during phorbol ester mediated constriction following application of SLIGRL.

4.2 METHODS

Experiments were performed according to section 2.3

4.3 RESULTS

Mouse mesenteric arteries, of average external diameter 222 ± 14μm, were pressurized to 40 mmHg for experimentation. 40mmHg proved to be a pressure at which vessels were near maximally inflated (figure 4.1), with pressures above 50 mmHg detrimentally effecting the vessel performance (data not shown).

4.3.1 SLIGRL mediated dilatation

Cumulative additions of SLIGRL (0.1-30μM) revealed a concentration-dependent dilatation of mouse mesenteric arteries, with near maximal relaxation achieved (dilatation at 30μM SLIGRL was 94.8 ± 0.74%, n=10) (figures 4.1 & 4.2). Incubation with L-NAME (100μM), caused a rightward shift of the concentration response curve, with an 80.8 ± 2.51% dilatation remaining at 30μM SLIGRL (n=10). Apamin (100nM) and TRAM-34 (1μM) were used to investigate the functional involvement of SK_{Ca} and IK_{Ca} respectively in L-NAME insensitve SLIGRL-mediated dilatations. Blockade of SK_{Ca} alone had no effect on SLIGRL mediated dilatation ($n=3$, $p>0.05$), whereas treatment with TRAM-34 inhibited the dilatation observed to SLIGRL ($n=3$, $p<0.05$). A small residual relaxation was still achieved to SLIGRL in the presence of 1μM TRAM-34 at higher concentrations of SLIGRL (10–30μM) which could be inhibited further, though not significantly, through the combined inhibition of SK_{Ca} and IK_{Ca} channels (n=6).

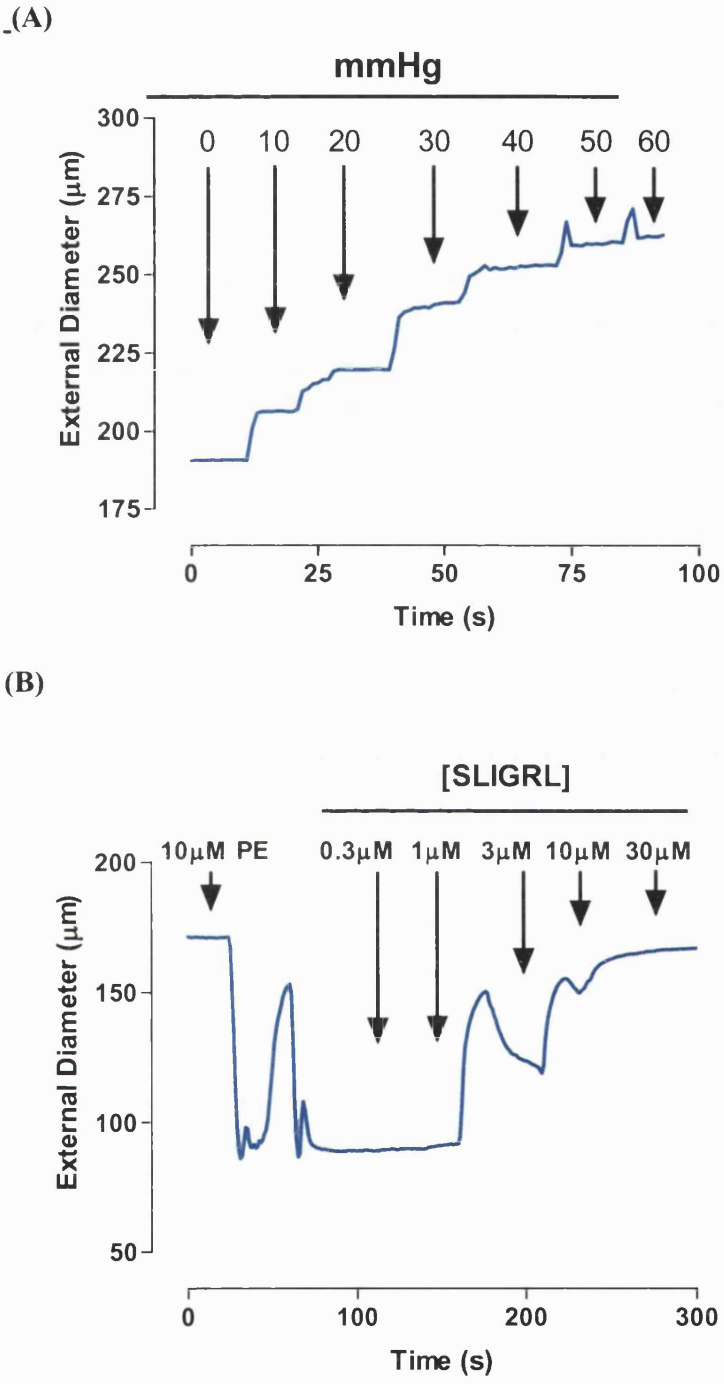

Figure 4.1; Dilatation of pressurized mouse mesenteric arteries by SLIGRL. (A) Passive inflation of cannulated pressurized mouse mesenteric arteries. (B) Representative trace demonstrating a concentration-dependent dilation of arteries with SLIGRL.

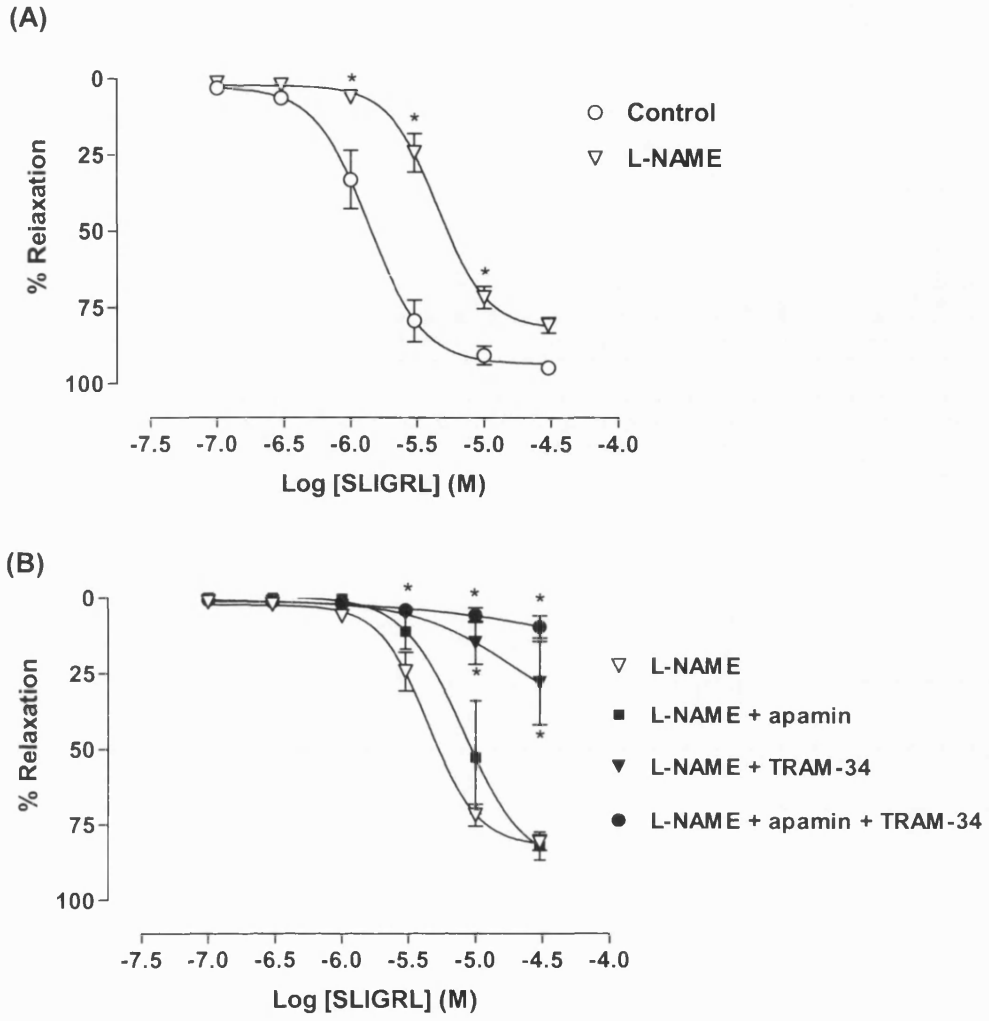

Figure 4.2; The involvement of K_{Ca} channels in the dilation of isolated mouse mesenteric arteries to SLIGRL. (A) Dilation to SLIGRL (control, n=10) persists in the presence of 100μM L-NAME (n=10). In the presence of L-NAME, or IK_{Ca} (1μM TRAM-34, n=3) channels partially inhibits the dilatation to SLIGRL, whereas inhibition of SK_{Ca} (100nM apamin, n=3) was without effect. Combined SK_{Ca} and IK_{Ca} inhibition further reduces the residual dilatation (n=6). (A) *denotes $p<0.05$ vs control, (B) *denotes $p<0.05$ vs L-NAME.

4.3.2 Cellular locations of SK_{Ca}, IK_{Ca} and BK_{Ca}

Immunohistochemistry revealed SK_{Ca} (figure 4.3) and IK_{Ca} (figures 4.4 & 4.5) channels to be expressed on endothelial cells of the rat cremaster. SK_{Ca} was expressed at adjacent endothelial cell borders and also in punctuate localised regions of the endothelial cell membrane, the punctate staining showed close correlation with the presence of holes in the internal elastic lamina. SK_{Ca} staining was absent from smooth muscle. IK_{Ca} was expressed in the perinuclear region in addition to punctuate localised regions of the endothelial cell membrane, which also showed close correlation with the presence of holes in the internal elastic lamina (figures 4.5 & 4.6). Endothelial cell IK_{Ca} staining appeared in some cases to pass into the smooth muscle side of the internal elastic lamina at holes in the internal elastic lamina, other punctate staining at sites of internal elastic lamina holes did not appear to be on the smooth muscle side of the internal elastic lamina (figure 4.6). BK_{Ca} staining was diffuse and non-specific in both smooth muscle and endothelial cells, indicative of non-specific background binding (figure 4.7).

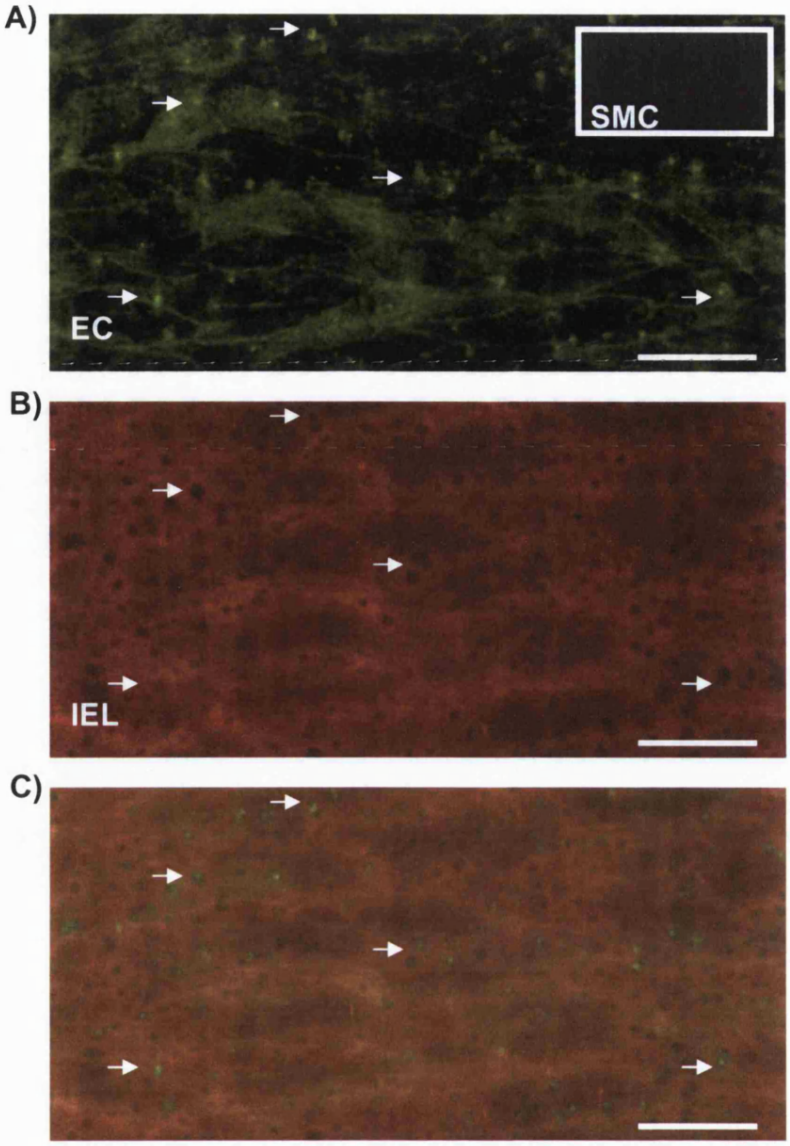

Figure 4.3; Expression of SK_{Ca} channels in mouse mesenteric arteries. Longitudinal vessel axis is left to right, with the long axis of endothelial cells (ECs) running horizontally and smooth muscle cells (SMCs) at ~90° to this axis. Cell layers were differentiated by mounting preparations media uppermost and optically sectioning with the confocal microscope from this surface. (A) Endothelial cell SK_{Ca} expression was confined to adjacent endothelial cell borders and localised punctate staining on the cell membrane (as indicated by white arrows), and was absent in smooth muscle cells (inset). (B) Autofluorescence shows a continuous internal elastic lamina (IEL) separating endothelial cells and smooth muscle cells, selected holes in the internal elastic lamina are marked with white arrows. (C) Overlayed SK_{Ca} staining and IEL autofluorescence demonstrates colocalisation (indicated by white arrows) of punctate SK_{Ca} staining and holes in the IEL. Scale bar (A-C) 50 μm.

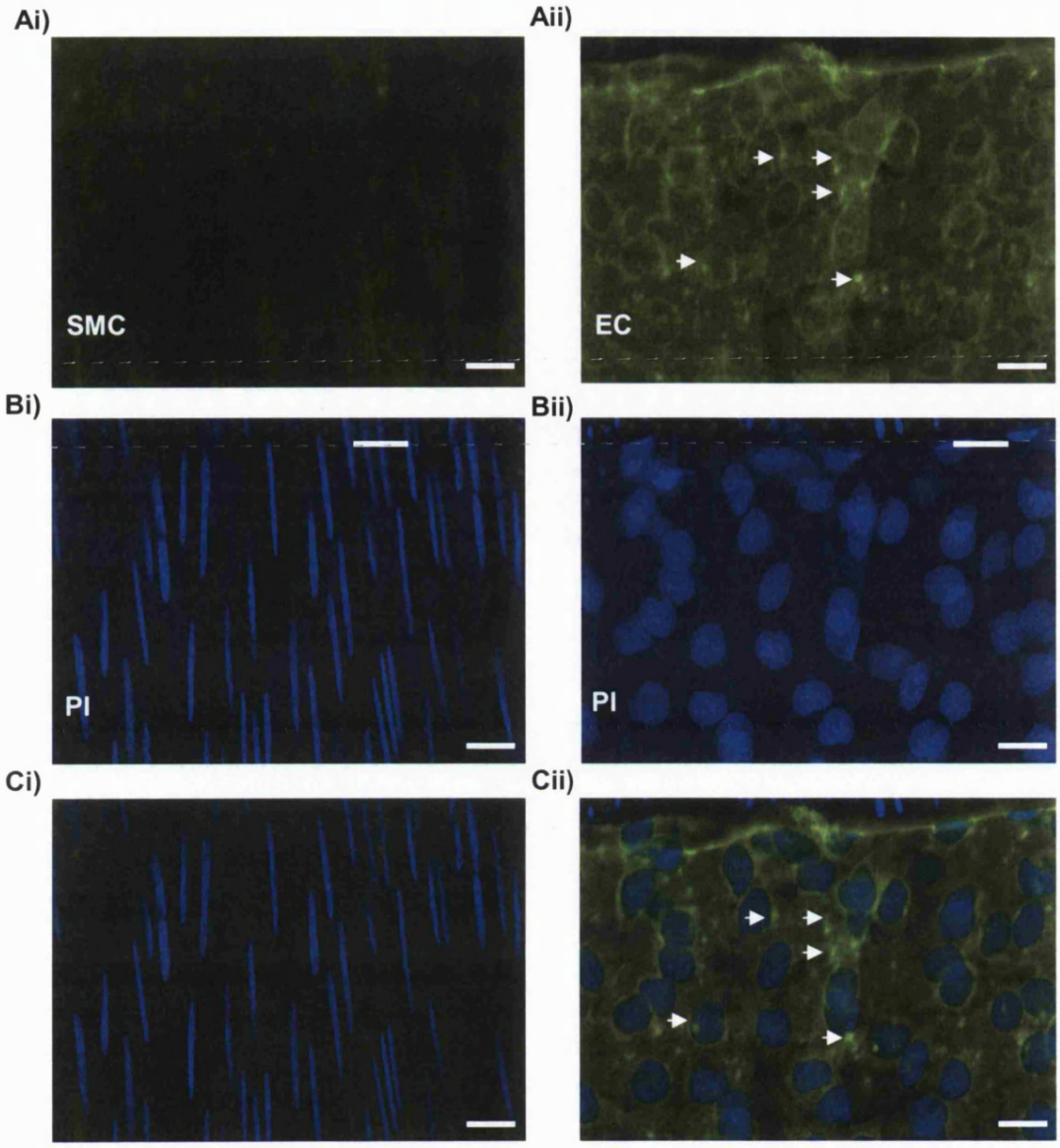

Figure 4.4; Expression of IK_{Ca} channels in mouse mesenteric arteries. Longitudinal vessel axis is left to right, with the long axis of endothelial cells (EC) running horizontally and smooth muscle cells (SMCs) at ~90° to this axis. Cell layers were differentiated by mounting preparations media uppermost and optically sectioning with the confocal microscope from this surface, additionally, nuclei were identified using propidium iodide (PI) staining, revealing long and thin smooth muscle cell nuclei (Bi) and ovoid endothelial cells (Bii). (Ai) smooth muscle cell IK_{Ca} expression was diffuse at a low level over the cell surface, indicative of non-specific background staining, whereas in endothelial cells (Aii) IK_{Ca} stained in the perinuclear region and also at localised punctate sections of the cell membrane (as indicated by white arrows). (Ci, Cii) Overlay of IK_{Ca} stain and PI images, punctate IK_{Ca} staining is indicated with white arrows. Scale bar (A-C) 50 μm.

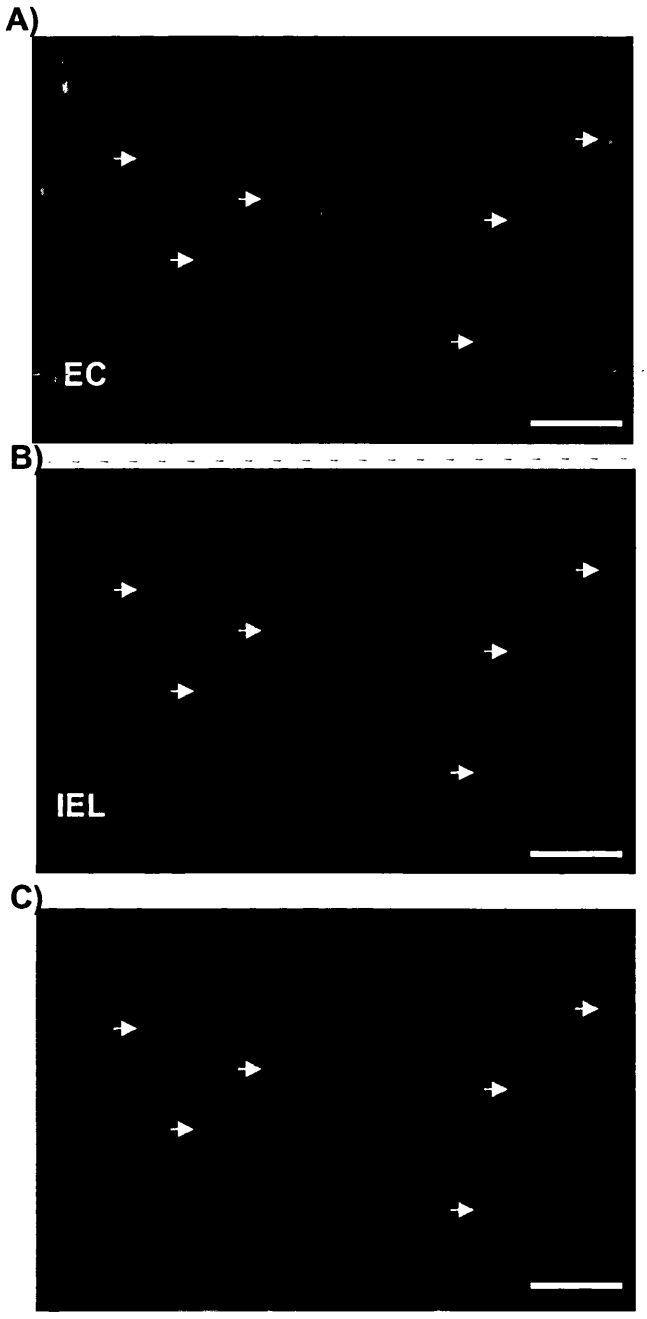

Figure 4.5; Expression of IK_{Ca} channels in mouse mesenteric arteries. Longitudinal vessel axis is left to right, with the long axis of endothelial cells (ECs) running horizontally. Cell layers were differentiated by mounting preparations media uppermost and optically sectioning with the confocal microscope from this surface. (A) Punctate endothelial cell IK_{Ca} expression was localised and punctate on the cell membrane (as indicated by white arrows). (B) Autofluorescence shows a continuous internal elastic lamina (IEL) separating endothelial cells and smooth muscle cells, holes in the IEL are marked with white arrows. (C) Overlayed SK_{Ca} staining and IEL autofluorescence demonstrates colocalisation (indicated by white arrows) of punctate IK_{Ca} staining and holes in the IEL. Scale bar (A-C) 50 μm.

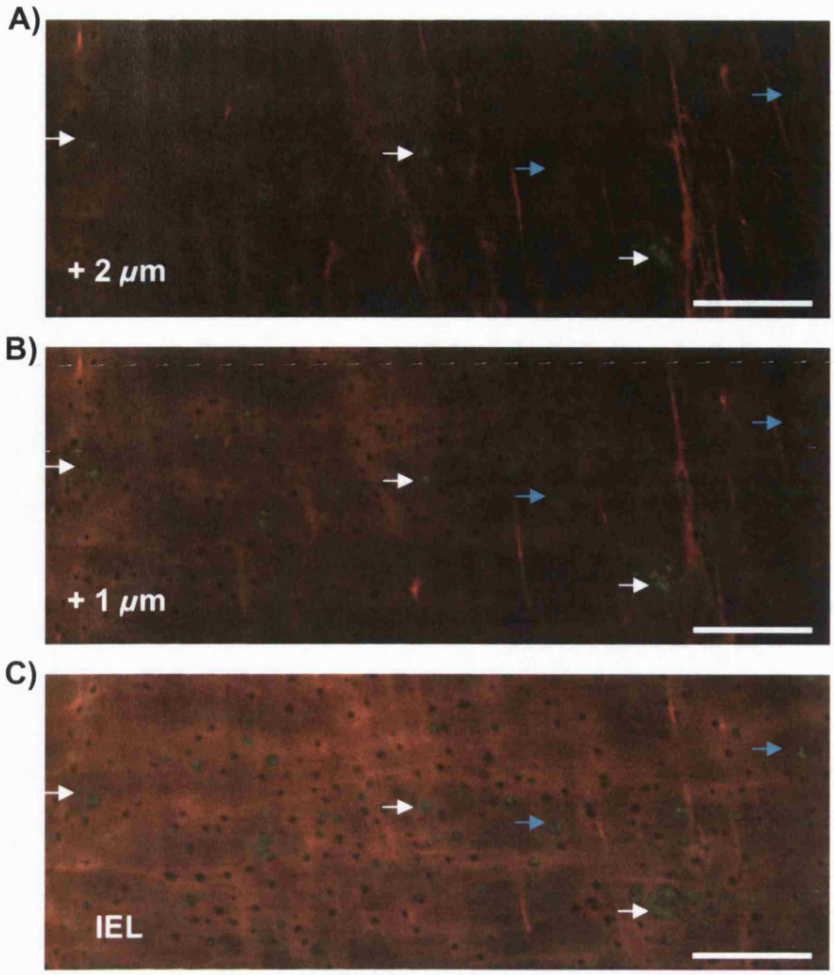

Figure 4.6; Expression of IK_{Ca} channels in relation to the IEL in mouse mesenteric arteries. Longitudinal vessel axis is left to right, with the long axis of endothelial cells running horizontally. Cell layers were differentiated by mounting preparations media uppermost and optically sectioning with the confocal microscope from this surface. (A) 2 μm and (B) 1 μm away from the IEL on the muscle side, punctate IK_{Ca} expression was detected at a higher intensity than that seen at the level of the IEL (C) (as indicated by white arrows). Other punctate IK_{Ca} staining was more prominent at the level of the IEL rather than on the muscle side of the IEL (as indicated by blue arrows). Scale bar (A-C) 50 μm.

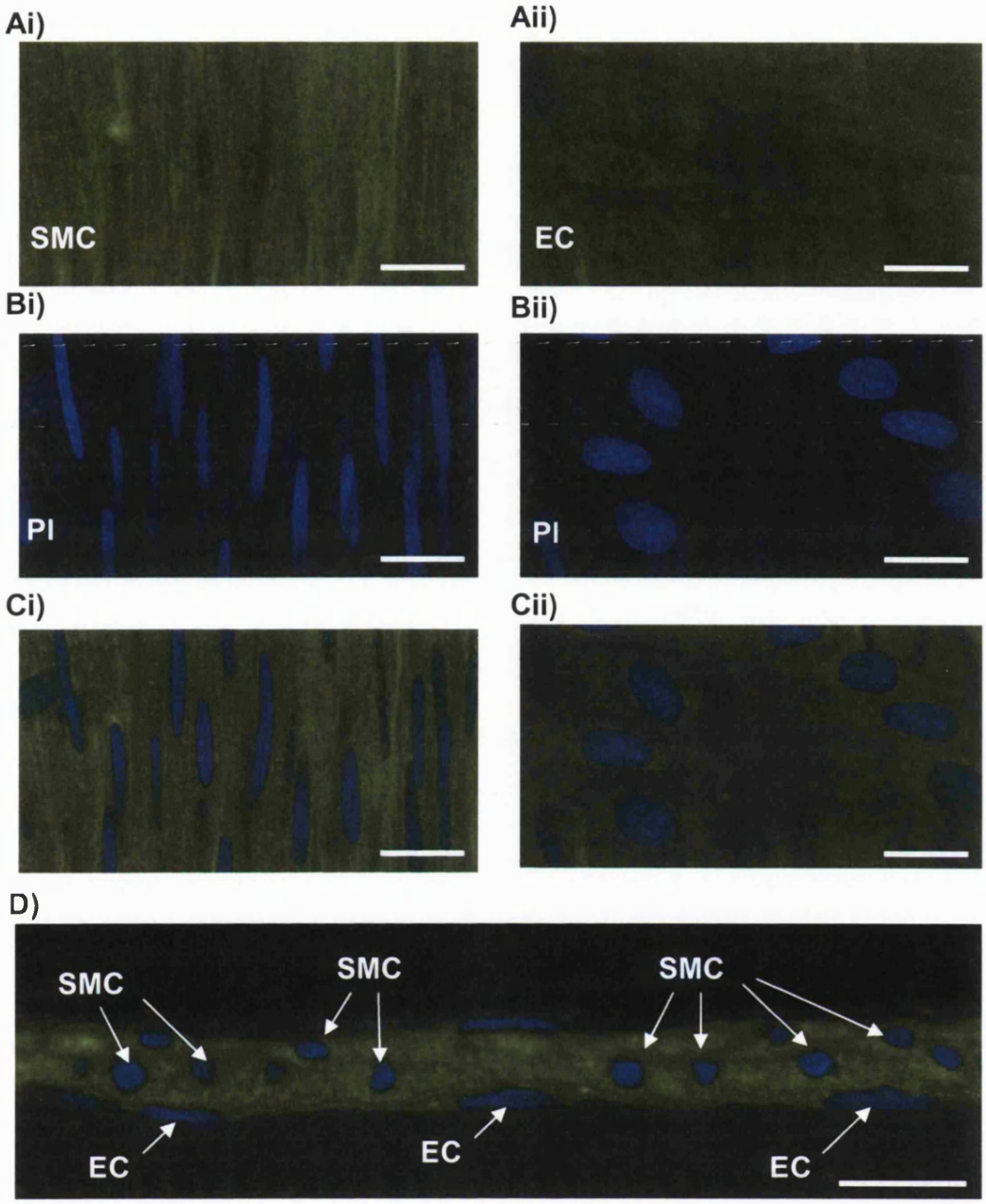

Figure 4.7; Expression of BK_{Ca} channels in mouse mesenteric arteries. Longitudinal vessel axis is left to right, with the long axis of endothelial cells (EC) running parallel and smooth muscle cells (SMCs) at ~90° to this axis (A-C). (Ai, Aii) endothelial cell and smooth muscle cell BK_{Ca} expression was diffuse at a low level over the cell surface, indicative of non-specific background staining. Cell layers were differentiated by mounting preparations media uppermost and optically sectioning with the confocal microscope from this surface, additionally, nuclei were identified using propidium iodide (PI) staining, revealing long and thin smooth muscle cell nuclei (Bi) and ovoid endothelial cells (Bii). (Ci, Cii) Overlay of BK_{Ca} stain and PI images. (D) Cross-section of BK_{Ca} and PI staining overlay in the vessel wall, in the cross-section, long and thin cell nuclei are endothelial and ovoid nuclei are smooth muscle nuclei. Low levels of diffuse staining are indicative of background staining in both cell types. Scale bar (A-D) 50 μm.

4.3.3 Phorbol esters and the EDHF response

Application of 100nM phorbol ester elicited sustained constrictions of mouse mesenteric arteries. Various concentrations of phorbol ester, in the range 1nM to 10μM, were tested for their ability to constrict the preparations, 100nM was selected as it gave a comparable level of constriction to the vessels as phenylephrine; the level of constriction to 100nM phorbol ester was 36.45% (n=3) of the maximal constriction, or 78.2% of the constriction response to 10μM phenylephrine (figure 4.8). High (10μM, n=10) and low (0.3μM, n=5) levels of preconstriction with phenylephrine were also demonstrated not to affect the dilatatory effectiveness of SLIGRL ($p>0.05$) (figure 4.8). In vessels constricted with 100nM phorbol ester, no relaxation to SLIGRL, in the range 0.1-30μM, was observed, indicating loss of the EDHF response.

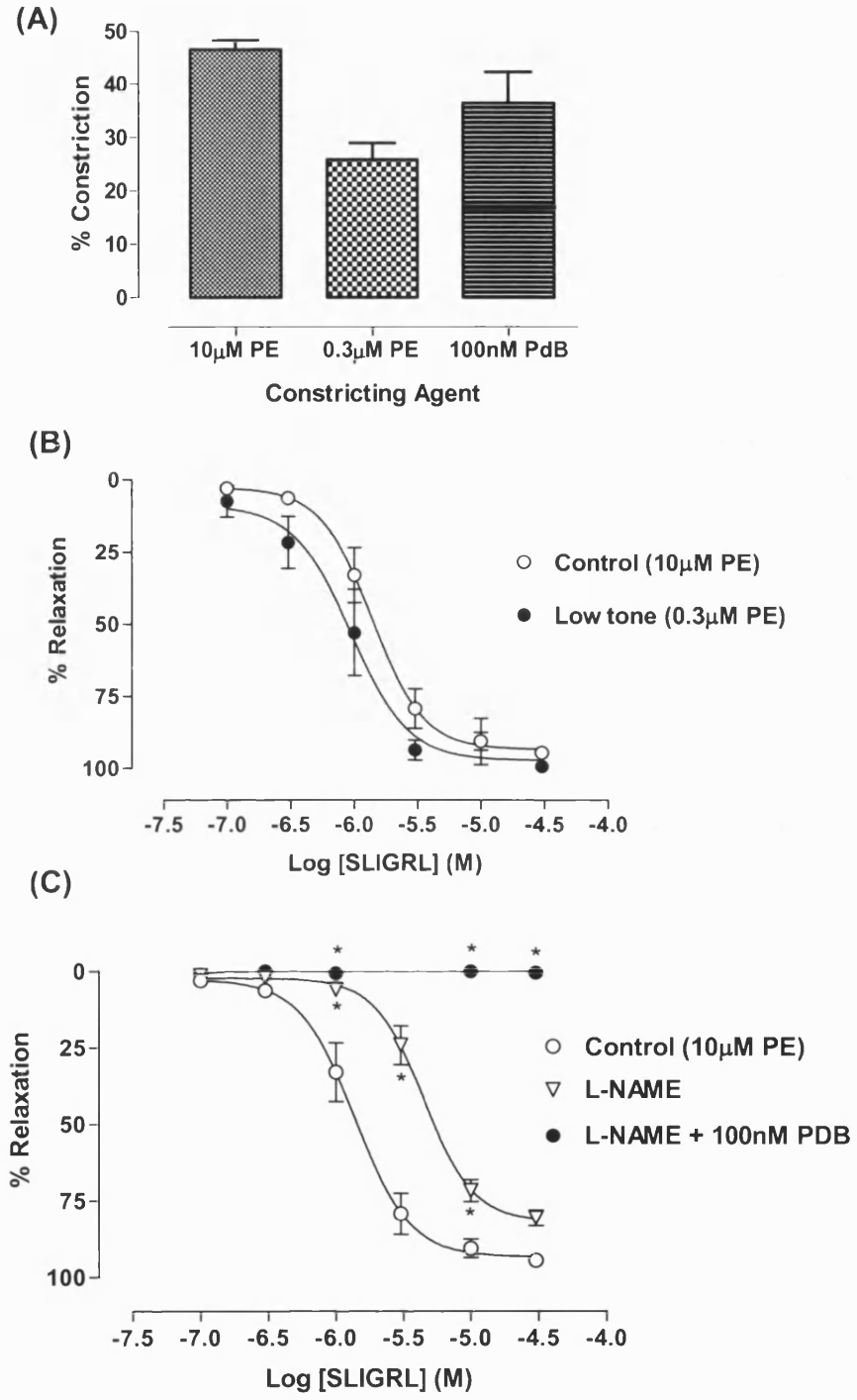

Figure 4.8; Responses to SLIGRL in the presence of phorbol ester in mouse mesenteric arteries. (A) The level of constriction induced by phenylephrine (10μM, n=10; 0.3μM, n=5) and 100nM phorbol ester (PDB, n=3). (B) High tone (10μM PE, n=10) and low tone (0.3μM PE, n=5) preconstruction did not affect vessel behaviour following application of SLIGRL. (C) Dilatation to SLIGRL persists in the presence of 100μM L-NAME (n=10), but is fully inhibited by 100μM L-NAME and 100nM phorbol ester (n=3). *denotes $p<0.05$ vs control.

4.4 DISCUSSION

Pressurized mouse mesenteric arteries exhibited NO-independent dilatatory responses following administration of the PAR2 selective ligand SLIGRL. The NO-independent relaxation was sensitive to IK_{Ca} blockade alone, but insensitive to SK_{Ca} blockade. The combined blockade of IK_{Ca} and SK_{Ca} channels appeared to further increase the block seen with IK_{Ca} inhibition alone. The results of this study confirm and build on the findings of previous studies which found the hyperpolarizing and relaxing effects of PAR-2 agonist administration to be inhibited by a combined treatment of apamin and charybdotoxin (Dora *et al.*, 2003; McGuire *et al.*, 2004). The present study utilises apamin and TRAM-34, to inhibit SK_{Ca} and IK_{Ca} respectively. TRAM-34 being a relatively new and highly selective IK_{Ca} inhibitor (Eichler *et al.*, 2003; Hinton *et al.*, 2003; Wulff *et al.*, 2000), whereas previously selective blockade of the IK_{Ca} channel was not possible, thus researchers were forced to use charybdotoxin which inhibits both IK_{Ca} and BK_{Ca} channels (Cowan *et al.*, 1993). The use of TRAM-34 allows us to ascertain definitively that BK_{Ca} plays no role in the EDHF mediated dilatation in the mouse cremaster, an important development considering that BK_{Ca} channels have been linked with EDHF type responses as a putative target of endothelium derived EETs. This observation is consistant with other studies which demonstrate a lack of functional BK_{Ca} in murine mesenteric arteries taken from mice of the age used in this study (Unpublished observation, Dr Kim Dora University of Bath). Furthermore, BK_{Ca} antibody staining of this vessel showed no specific staining, in either endothelium or smooth muscle, with the efficacy of the anti-BK_{Ca} anitbody verified in other studies (personal communication Dr Shaun Sandow, Dr Kim Dora) this result indicates that BK_{Ca} is not expressed in these vessels.

Immunohistochemistry of IK_{Ca} and SK_{Ca} revealed these channels to be localised to the endothelium, increases in endothelial cell $[Ca^{2+}]_i$ following SLIGRL administration would therefore lead to activation of these receptors resulting in hyperpolarization of the endothelium, rather than these channels as targets for a putative EDHF to act at on the vascular smooth muscle. The identity of the EDHF remains elusive from this study, although the activation of endothelial K_{Ca} appears vital for its production. Although H_2O_2 has been demonstrated to hyperpolarize human endothelial cells via activation of endothelial K_{Ca} (Bychkov *et al.*, 1999), inhibition of those channels only partially reduces the proposed H_2O_2 mediated EDHF response (Matoba *et al.*, 2000), in contrast to the data presented in this study where blockade of the endothelial K_{Ca}, SK_{Ca} and IK_{Ca}, fully inhibited EDHF responses making H_2O_2 an unlikely EDHF in this instance. The abolition of EDHF responses by blockade of endothelial K_{Ca} has previously been described in studies of EDHF pathways in mouse mesenteric arteries. From those studies, the authors propose the involvement of hyperpolarization transfer from endothelium to smooth muscle via myoendothelial gap junctions (Dora *et al.*, 2003), or Ba^{2+}/ouabain sensitive K_{IR} and Na^+/K^+-ATPase activation (McGuire *et al.*, 2004) as part of the EDHF mechanism. The two hypotheses are not necessarily mutually exclusive and indeed may work synergistically.

The punctate staining of both SK_{Ca} and IK_{Ca} at sites corresponding to holes in the internal elastic lamina would appear to support an intricate interplay between myoendothelial gap junctions and localised areas of high K_{Ca} expression. Electron microscopy studies of resistance artery morphology typically reveal that in addition to myoendothelial gap junctions many projections protrude from the endothelium at the sites of holes in the internal elastic lamina and

do not form a typical pentalaminar plaque associated with gap junctions (personal communication, Dr Shaun Sandow, University of Bath). It is an attractive hypothesis to suggest that the expression pattern of SK_{Ca} and IK_{Ca} at holes in the internal elastic lamina serves as a mechanism of avoiding the diffusion barrier that the internal elastic lamina may represent, the apparent staining of endothelial IK_{Ca} on the smooth muscle side of the internal elastic lamina is testimony to this fact. It is feasible to imagine a situation whereby projections of the endothelium through the internal elastic lamina are regions heavily expressing SK_{Ca} and IK_{Ca} and that these projections may act as 'pseudosynapses', substances released from them would rapidly act at the neighbouring smooth muscle. Alternatively K_{Ca} may regulate intact myoendothelial gap junctions through as yet undefined microdomain signalling mechanisms. Not every hole in the internal elastic lamina will have projections, complete or otherwise, running through it, and it may be that the holes with concommitant staining are exclusively those holes which do not have any projections running through them. In this instance the diffusion of K^+ to smooth muscle from the endothelium may be eased by the removal of the hypothetical diffusion barrier of the internal elastic lamina.

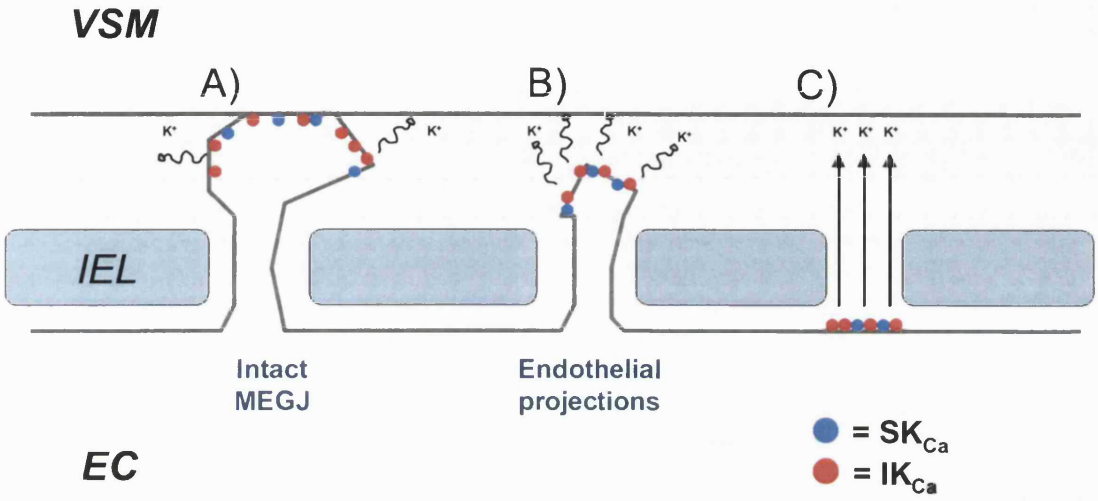

Figure 4.9; Correlation of SK_{Ca} and IK_{Ca} expression with holes in the internal elastic lamina (IEL). A) SK_{Ca} and IK_{Ca} may be expressed on intact myoendothelial gap junctions, perhaps modulating their function or acting as a site for K^+ discharge proximal to the vascular smooth muscle (VSM). B) Endothelial cell (EC) projections with high levels of SK_{Ca} and IK_{Ca} may represent 'pseudosynapses', providing K^+ release site which is close to the vascular smooth muscle. C) If no projections from the endothelium are present, colocalisation of SK_{Ca} and IK_{Ca} with holes in the IEL may be a mechanism of avoiding the diffusion barrier that the IEL may represent to the diffusion of K^+ from the endothelium to vascular smooth muscle.

Throughout the current investigation SLIGRL proved to be an activator of the mouse mesenteric endothelium, and PAR2 activation resulted in both NO-dependent and NO-independent relaxations characteristic of an EDHF response. The apparent inactivity of SLIGRL on vascular smooth muscle possibly reflects a low-level of expression of PAR2 on the underlying smooth muscle as activation of PAR2 on vascular smooth muscle has previously been demonstrated to generate a constrictor response in mouse renal arteries (Moffatt *et al.*, 1998), presumably through elevation of smooth muscle cell Ca^{2+}. Alternatively the lack of smooth muscle response to PAR2 activation may be due to a functional association of PAR2 to another receptor expressed in the muscle (see Cicala, 2002). The actions of SLIGRL have previously been confirmed to be specific to the PAR2 receptor in a receptor knockout mouse study (McGuire *et al.*, 2002). Other studies from our laboratory (unpublished observations Dr Alister McNeish, Nicola Gallagher and Dr Hiromichi Takano) indicate that SLIGRL is devoid of action in de-endothelialised mouse mesenteric arteries, further suggesting that relaxations mediated through its use are endothelium dependent.

The binding of a PAR agonist to a receptor is irreversible once the receptor is cleaved, in spite of this PARs may still play an important physiological role if there is a high turnover of receptors or if only a small receptor occupancy is required to generate a significant response. Furthermore, PARs are not only expressed in healthy tissue but also in atherosclerotic plaques and after vascular injury, indicating an important role in inflammatory processes and response to injury (Patterson *et al.*, 2001). Thus PARs may represent an important physiological and/or pathophysiological endothelium activating mechanism. Furthermore, the differential

expression of PAR receptor subtypes in the vasculature could be advantageous for therapeutic manipulation.

The use of phorbol ester, the DAG mimetic, to preconstrict vessels, abolished the EDHF response to SLIGRL, inspite of the retained ability of the peptide to elicit smooth muscle cell hyperpolarization (unpublished observations, Dr Hiromichi Takano and Nicola Gallagher, University of Bath). Although phorbol esters do depolarize upon constriction they do not do so to such a magnitude as a similar strength phenylephrine induced constriction (unpublished observations, Dr Hiromichi Takano and Nicola Gallagher University of Bath), this observation coupled with the loss of the EDHF response suggests that phorbol esters in part cause constriction through mechanisms independent of smooth muscle depolarization and increased Ca^{2+} entry through voltage gated Ca^{2+} channels, i.e. by Ca^{2+} sensitization. The most prominent intracellular target of DAG and phorbol esters is protein kinase-C, but DAG and phorbol esters can also activate other pathways, including DAG kinase and protein kinase-D, so some caution should be exercised in interpreting results obtained with phorbol esters (Brose *et al.*, 2002). The consequences of PKC activation in the vasculature are multiple as the enzyme family is involved in a host of intracellular signalling pathways, those of relevance to vascular function include the activation of phospholipase-A_2 with increased production of arachidonic acid metabolites and the inhibition of Na^+/K^+-ATPase (Koya *et al.*, 1998), also of importance is the sensitization of contractile proteins by PKC (Sato *et al.*, 1992).

The lack of EDHF responses following SLIGRL administration in phorbol ester constricted vessels, may be of interest in the study of endothelial dysfunction. Upregulation of the PKC

pathway has been linked to the development of diabetic complications, with increased transcription, translation, and activity of PKC seen in early stages of experimental diabetes (Guo *et al.*, 2003; Hempel *et al.*, 1997), however the mechanism of this upregulation by hyperglycaemia remains elusive. Given that there was no EDHF-type functional response seen during phorbol ester induced contraction, it is feasible that the loss of effectiveness of EDHF-like mechanisms may be related, at least in part, to the vascular complications associated with diabetes. Whilst an interesting hypothesis, it may be flawed as the mechanisms of PKC dependent constrictions appear to differ in diabetic vs control vessels (Hattori *et al.*, 1995), with constrictions in healthy vessels likely due to sensitization and those in diabetic vessels due to Ca^{2+} influx through voltage gated Ca^{2+} channels, thus the reverse hypothesis may be true – that EDHF is of greater functional significance in endothelial dysfunction.

4.4.1 Summary

The clarification of the K_{Ca} subtypes involved in EDHF-like dilatations in this study improves upon previous investigations which relied on non-selective inhibition of IK_{Ca} channels with charybdotoxin, with the use in its place of the more selective TRAM-34. Furthermore, immunohistochemistry of K_{Ca} subtypes revealed that both subtypes involved in EDHF mediated dilations were situated exclusively on the endothelium in mouse mesenteric arteries. Expression of SK_{Ca} and IK_{Ca} correlated with holes in the internal elastic lamina, an observation which potentially links SK_{Ca} and IK_{Ca} with a regulatory role in gap junction communication or represents a specialised signalling mechanism where by endothelial K^+ efflux can access

vascular smooth muscle without obstruction from the postulated diffusion barrier of the internal elastic lamina.

Constrictions due to phorbol ester application were resistant to EDHF-like relaxation, indicating the constriction observed is probably due to the depolarization independent constricting mechanisms of phorbol esters such as Ca^{2+} sensitization. The loss of EDHF responses in instances where PKC activity is increased, for example in diabetes, may be of physiological and pathophysiological consequence.

ACKNOWLEDGEMENTS

Immunohistology studies presented in this chapter were performed with the assistance of Dr Kim Dora.

Chapter Five

EDHF dilatations in rat isolated cremaster arteries involve SK_{Ca} IK_{Ca} and BK_{Ca} channels

5.1 INTRODUCTION

In rat cremaster arteries, the endothelium-dependent dilatation to ACh can be fully inhibited in the combined presence of a NO synthase inhibitor and raised extracellular K^+ (Bakker *et al.*, 1997), showing important roles for both NO and EDHF in driving smooth muscle relaxation. The dilatation to ACh follows a rise in endothelial cell $[Ca^{2+}]_i$ (Falcone *et al.*, 1993), and smooth muscle cell hyperpolarization (Kotecha *et al.*, 2005). The hyperpolarization is known to involve opening of K_{Ca} channels (Bakker *et al.*, 1997), yet the K_{Ca} channel subtypes and cell-specificity are not established.

The EDHF response has been extensively studied in many resistance arteries, with at least three pathways suggested to lead to smooth muscle cell hyperpolarization, and being dependent on activation of K_{Ca} channels. In general, the SK_{Ca} and IK_{Ca} channels are present on the endothelium, whereas the BK_{Ca} channels are localized to the surrounding muscle. Hyperpolarization of the endothelium through activation of endothelial K_{Ca} channels can be transferred to the inner smooth muscle cell layer via myoendothelial gap junctions (Emerson *et al.*, 2000; Sandow *et al.*, 2002) without necessarily involving the opening of smooth muscle K^+ channels, to account for EDHF activity. In addition to this pathway, the release and accumulation of K^+, perhaps at specialized myoendothelial regions, can act to hyperpolarize the underlying smooth muscle by inducing current through K_{IR} and an increased activity of the electrogenic Na^+/K^+-ATPase (Edwards *et al.*, 1998). Both of these mechanisms may play an integral role in EDHF activity by working in conjunction, as is evident in the rat mesenteric artery (Mather *et al.*, 2005). In other arteries, such as coronary and renal vessels, CYP-450

metabolites have been shown to be of importance in the EDHF response (for reviews see (Campbell *et al.*, 1999; Roman, 2002)). In these vessels, EETs released through the metabolism of arachidonic acid may activate BK_{Ca} localized to the muscle (Campbell *et al.*, 1996), and potentially also directly activate endothelial cell K_{Ca} (Edwards *et al.*, 2000). Additionally, in an *in vitro* model system using cultured endothelial cells from human umbilical vein and porcine coronary artery, it has been suggested that EETs may facilitate electrotonic spread of hyperpolarization through and from the endothelium (Popp *et al.*, 2002).

Importantly, the luminal pressure-mediated depolarization of smooth muscle influences the ability of a given hyperpolarization to generate an increase in diameter, with a pressure near 75mmHg optimizing the dilatory response (Kotecha *et al.*, 2005). A transmural pressure of 75mmHg also represents a pressure that is physiologically relevant in this vessel (Hill *et al.*, 1992) and is associated with the generation of a robust spontaneous myogenic response. Since in most arteries the EDHF response to ACh is thought to be due to activation of endothelial cell K_{Ca} channels, and there is some evidence for a role for CYP-450 metabolites in the response of the rat cremaster artery (Bakker *et al.*, 1997), we chose to characterize the EDHF response further at this physiological pressure. The dilatory reponse to ACh was characterized through the use of subtype selective K_{Ca} channel inhibitors and correlated with vessel morphology using electron microscopy.

5.2 METHODS

Experiments were performed according to section 2.4

5.3 RESULTS

Rat cremaster muscle arteries, with passive internal diameters in the range of 110–190μm, developed spontaneous myogenic tone so that vessels were typically constricted to at least 50% of their maximum diameter (table 5.1 and figure 5.3).

5.3.1 ACh mediated increases in endothelial cell $[Ca^{2+}]_i$

Vessels were successfully loaded via the lumen with the Ca^{2+}-sensitive dye fluo-4 AM, resulting in ~100% endothelial cell loading, with minimal loading of smooth muscle cells (figure 5.1). Random, basal oscillations in endothelial cell Ca^{2+} were frequently observed, and generally only a subset of cells responding at a given time. Application of ACh (10nM-3μM) stimulated marked concentration dependent increases in fluorescence in all cells, which oscillated in most cells, yet the average fluorescence intensity of 16 cells had dampened oscillations, suggesting the oscillations were asynchronous (figure 5.1, representative of 3 similar experiments). The range of concentration-dependent rises in Ca^{2+} correlated well with dilatation (figure 5.1C). Endothelial cell Ca^{2+} continued to increase with high concentrations of ACh (3-30μM ACh), even in excess of concentrations which evoked maximal dilatation. A closer inspection of the response to 0.1μM ACh revealed that the peak rise in endothelial cell Ca^{2+} was asynchronous between adjacent cells (figure 5.2A & B) and passed as a repeating wave along the cells (figure 5.2A & C). Synchronized oscillations in endothelial cell Ca^{2+} were never observed, even at the highest concentrations of ACh.

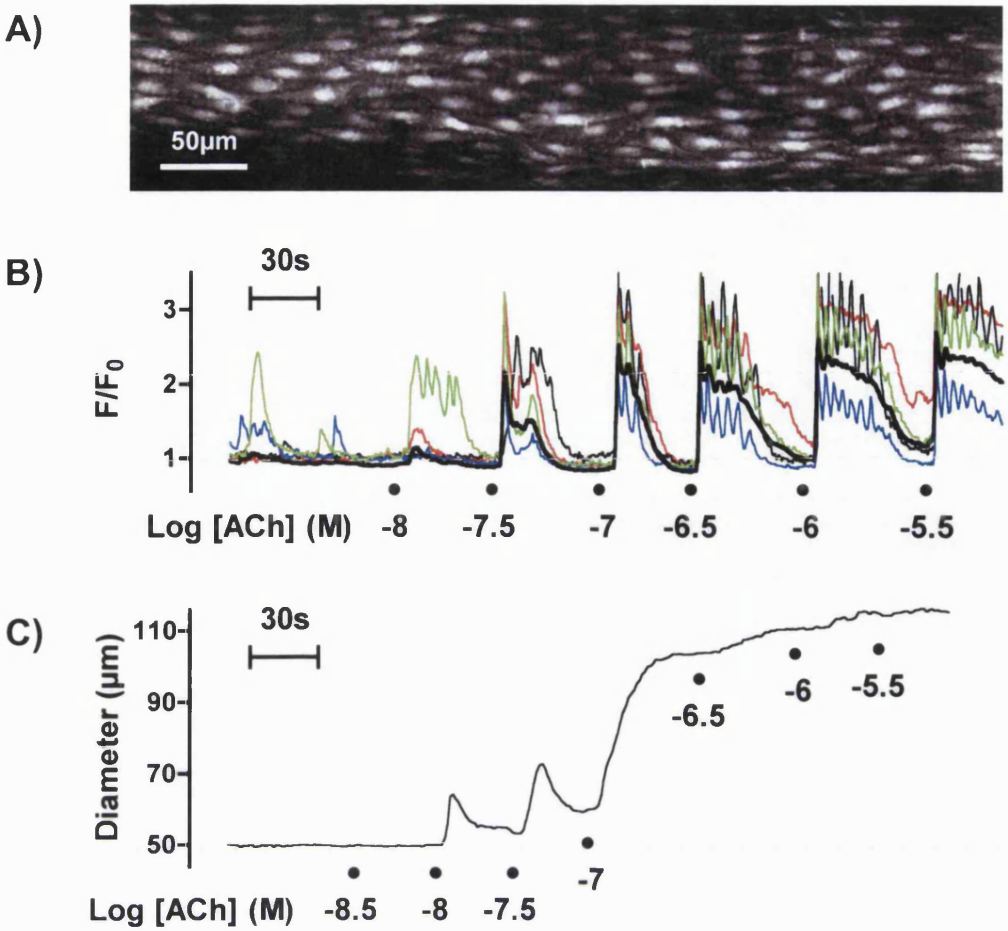

Figure 5.1; Representative images and traces of endothelial cell Ca^{2+} and diameter measurements. (A) An artery luminally-loaded with fluo-4 dye shows selective endothelial cell loading. Representative traces for cumulative ACh concentration response curves for changes in endothelial cell Ca^{2+} (B) and diameter (C). (B) The fluorescence intensity of 16 randomly picked entire cells was averaged (thick black line). The thin lines show the responses from 4 of these entire cells. Note that the magnitude of Ca^{2+} oscillations is greater within a given cell than the averaged value.

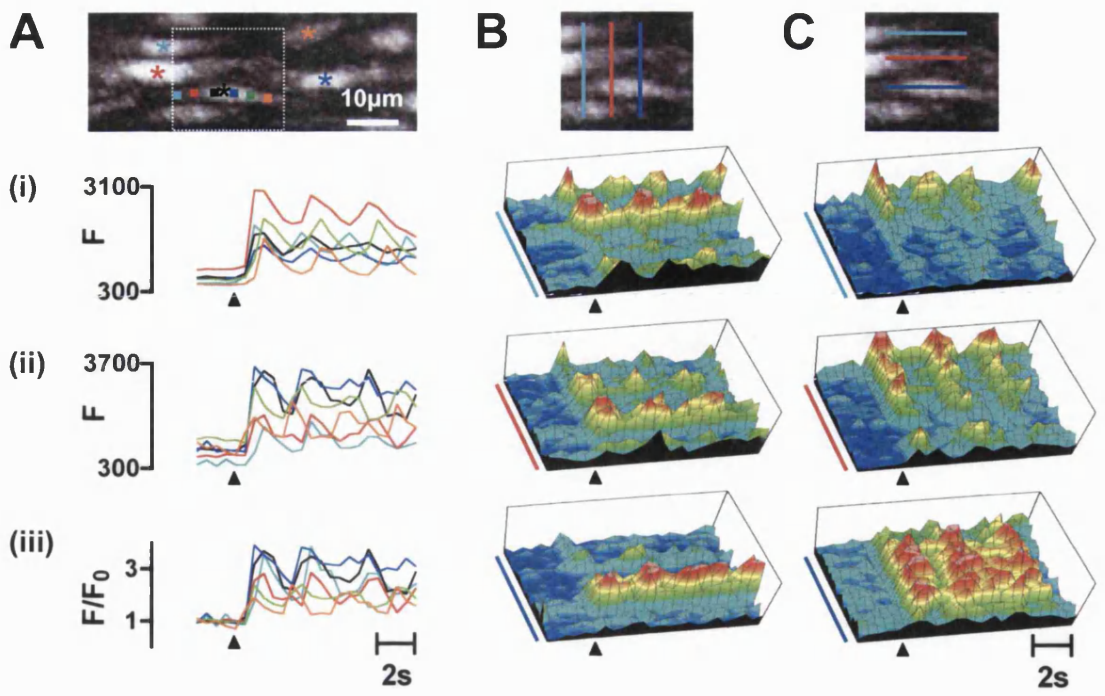

Figure 5.2; Dynamics of endothelial cell Ca^{2+} response to 0.1μM ACh. (A) The section of artery depicted in the white box shown in Figure 1A was analyzed in more detail. (i) The average fluorescence intensity of the entire cells indicated by the coloured asterisks in A. Note that the peaks in fluorescence are not synchronous between adjacent cells. The average fluorescence intensity (ii) and relative fluorescence intensity (iii) of the regions within a single endothelial cell indicated by the coloured boxes in A. (B,C) Enlarged image of the region covered by a dashed white box in A. The time course of fluorescence intensity in the regions indicated by the vertical (B) and horizontal (C) lines. The vertical scale is 0 to 4100 units of fluorescence intensity, represented by the colour range black to pink. In all panels 0.1μM ACh was added at the time indicated by the arrow.

5.3.2 ACh mediated dilatation

Cumulative addition of ACh (1nM-3μM) evoked concentration-dependent dilatation of the cremaster artery (Figure 5.3), with 3μM ACh stimulating 95.4 ± 0.8% (near maximal) dilatation (EC_{50} = $5.6x10^{-8}$, n = 37) (Figure 5.4A). Incubation with the nitric oxide synthase inhibitor, N^G-nitro-L-arginine methyl ester (L-NAME, 100μM), caused a small rightward shift of the concentration response curve, with 83.5 ± 3.6% maximal dilatation remaining at 3μM ACh (n = 10). In the presence of L-NAME, increasing the extracellular K^+ concentration from 4.7 to 35mM (and thereby preventing cellular hyperpolarization) fully inhibited ACh-mediated dilatation (n = 3). Passing an air bubble through the lumen of arteries ablated vasodilatation to ACh (without inhibiting myogenic tone, n = 3, data not shown), confirming the endothelium-dependence of the dilatation to ACh.

5.3.3 Role of SK_{Ca}, IK_{Ca} and BK_{Ca} channels in ACh mediated dilatation

The role of SK_{Ca}, IK_{Ca}, and BK_{Ca} channels in the L-NAME-insensitive, ACh-mediated, dilatation was investigated with subtype selective inhibitors (Figure 5.4B). TRAM-34 (1μM) and apamin (100nM) and were used to investigate the involvement of IK_{Ca} and SK_{Ca}, respectively. Blockade of IK_{Ca} or SK_{Ca} alone had no effect on ACh-mediated dilatation ($p>0.05$, both n = 3, data not shown), whereas treatment with TRAM-34 and apamin in combination significantly reduced dilatation to ACh ($p<0.05$, n = 8), leaving a residual dilatation of 61.8 ± 8.2% with 3μM ACh. Selective blockade of the BK_{Ca} channel (100nM iberiotoxin) inhibited the residual relaxation with 1 and 3μM ACh in the presence of TRAM-

34 and apamin ($p<0.05$, n = 6, Figure 5.5A). In contrast, inhibition of BK_{Ca} channels alone did not effect the L-NAME resistant dilatation ($p>0.05$, n = 7, Figure 5.4B).

5.3.4 Role of K_{IR} channels and the Na^+/K^+-ATPase in ACh mediated dilatation

The role of the K_{IR} and the Na^+/K^+-ATPase were investigated by selective inhibition with 30μM Ba^{2+} and 10μM ouabain, respectively. Relaxation was stimulated to exogenous isotonic 15mM K^+ (Figure 5.4C), evoking 61.4 ± 6.6% dilation (n = 5). The relaxation to 15mM K^+ was reduced by Ba^{2+} ($p<0.05$, n=3) to 32.3 ± 8.1%, but unaffected by ouabain ($p>0.05$, n = 3). Nearly complete inhibition of K^+ induced dilatation was achieved in the presence of both Ba^{2+} and ouabain ($p<0.05$, n = 6), only 2.0 ± 7.1% remaining. This combination of blockers (in the presence of L-NAME) did not alter the ACh-evoked dilatation ($p<0.05$, n = 5) (Figure 5.4B).

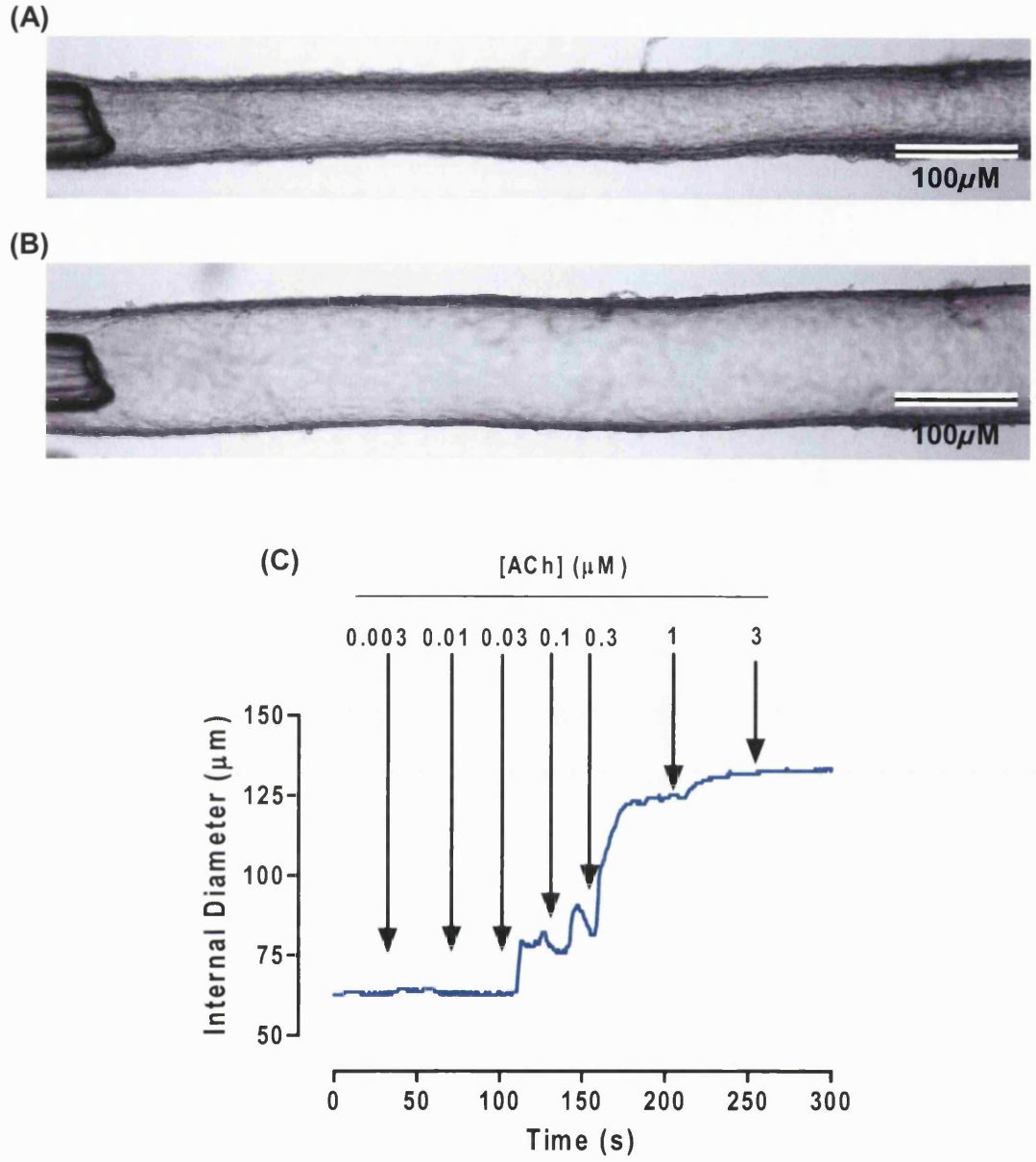

Figure 5.3; Dilatation of myogenically constricted pressurized rat cremaster arteries by ACh. (A) Pressurized arteries exhibit spontaneous myogenic tone resulting in a stable and uniform contraction. (B) Vessels maximally relax following administration of ACh. (C) Typical response of pressurized rat cremaster arteries to cumulative additions of ACh.

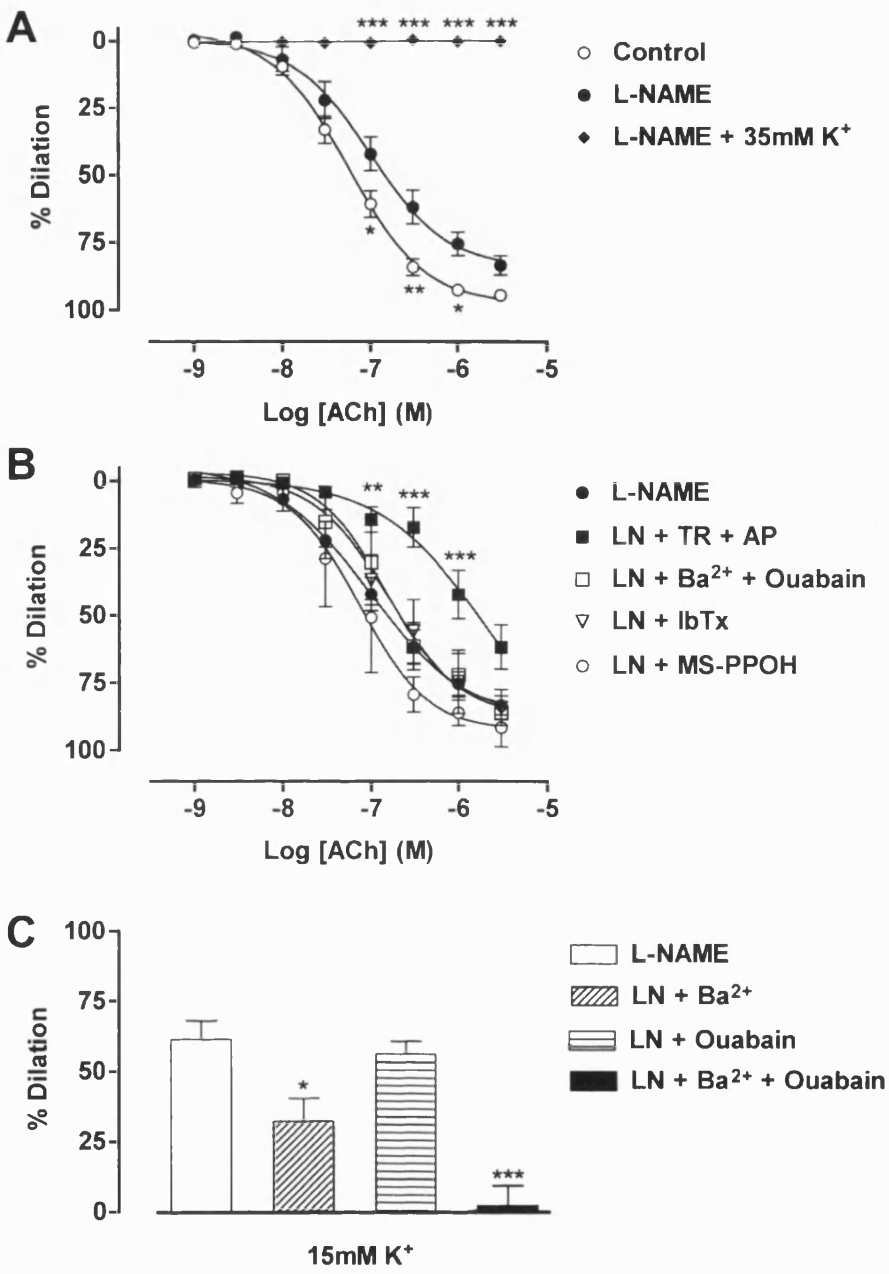

Figure 5.4; The involvement of K^+ channels in the dilation of isolated rat cremaster arterioles to ACh. (A) Dilation to ACh persists in the presence of 100μM L-NAME (n = 10), but is fully blocked by the additional presence of raised extracellular K^+ (35mM, n = 3). (B) In the presence of L-NAME (LN), inhibition of IK_{Ca} and SK_{Ca} channels with TRAM-34 (1μM, TR) and apamin (100nM, AP), respectively, partially inhibits the dilation to ACh (n = 8). Inhibition of K_{IR} and Na^+/K^+-ATPase with Ba^{2+} (30μM) and ouabain (10μM), respectively, had no effect on L-NAME insensitive ACh-mediated dilations (n = 5). Selective inhibition of BK_{Ca} channels using iberiotoxin (IbTx, 100nM, n = 7) or of CYP-450 using MS-PPOH (10μM, n = 3) also had no effect. Note that L-NAME alone data are repeated in panels A and B. (C) The combined presence of Ba^{2+} and ouabain was necessary to fully block relaxation stimulated by 15mM K^+ (isotonic, final bath concentration, n = 5). None of these treatments had a significant effect on basal tone (Table 1). *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$ vs L-NAME.

5.3.5 Role of EETs and cytochrome P-450

The potential role of CYP450-derived metabolites in L-NAME resistant ACh-induced dilatation was investigated (Figure 5.5). Incubation in 10μM MS-PPOH (CYP450 epoxygenase specific inhibitor) did not affect dilatation to ACh ($p>0.05$, n = 3, Figure 5.4B). However the residual dilatation in the presence of TRAM-34 and apamin, could be reduced by either 20μM 17-ODYA (a non-specific CYP450 inhibitor) or MS-PPOH ($p<0.05$, both n = 3) (Figure 5.5A), mimicking the effect of BK_{Ca} blockade. Furthermore, application of 11,12-EET, a metabolite of the CYP450 epoxygenase pathway, evoked concentration-dependent dilatation (Figure 5.5B). 10μM 11,12-EET stimulated 67.0 ± 6.8% of maximum dilatation (n = 8), which was markedly attenuated by BK_{Ca} blockade to 4.9 ± 6.6% of the maximum dilatation (n = 4). However, 14,15-EET, another EET isomer generated by the CYP450 epoxygenase, had no effect in this vessel, at concentrations up to 10μM (n = 3).

5.3.6 Effect of 11,12-EET on endothelial cell $[Ca^{2+}]_i$

11,12-EET (10μM) did not alter endothelial cell $[Ca^{2+}]_i$ in preparations where 1μM ACh stimulate a robust increase (Figure 5.5C). The ability of hyperpolarization to stimulate changes in endothelial cell $[Ca^{2+}]_i$ was tested further. The activator of K_{ATP}, levcromakalim (1μM), stimulated maximum dilatation (98.4 ± 1.0%, n = 4), but did not affect endothelial cell $[Ca^{2+}]_i$ (Figure 5.5C). The dilatation to levcromakalim was fully blocked by raised extracellular KCl (35mM) (n = 4), supporting an involvement of K^+ efflux in the response. In addition, raised

extracellular KCl (35mM) did not alter the magnitude or time-course for the rise in endothelial cell $[Ca^{2+}]_i$ to 1μM ACh (n = 3, p>0.05) (Figure 5.5C).

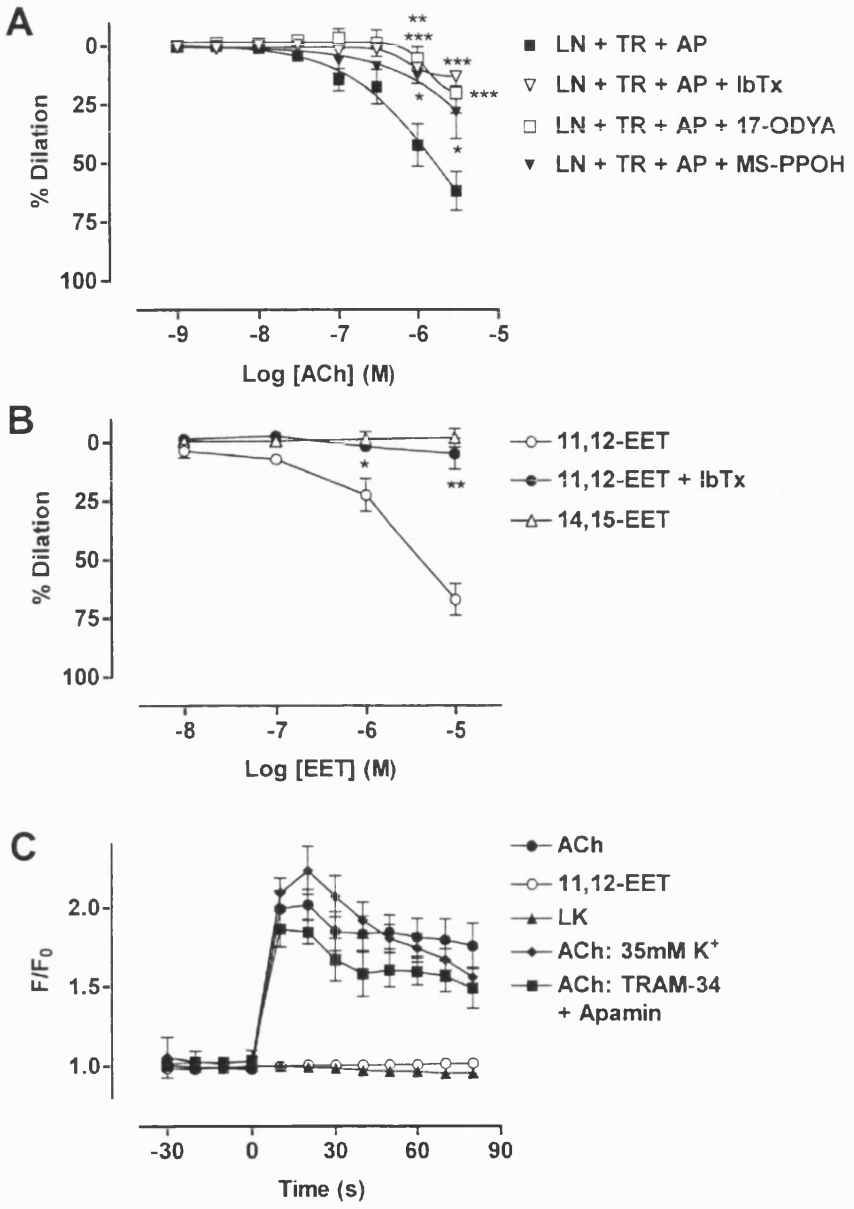

Figure 5.5; Contribution of CYP-450 to the EDHF response. (A) Inhibition of BK_{Ca} channels using iberiotoxin (IbTx, 100nM) (n = 6) or of CYP-450 (10μM MS-PPOH or 20μM 17-ODYA, each n = 3) caused further inhibition of the ACh mediated dilation persisting in the presence of IK_{Ca} and SK_{Ca} blockade (1μM TRAM-34 (TR) and 100nM apamin (AP), respectively). Note that LN + TR + AP data are the same as in Figure 3. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$ vs L-NAME. (B) In the presence of L-NAME (100μM), application of exogenous 11,12-EET caused concentration dependent dilation of cremaster vessels (n = 8) that could be almost fully attenuated by blockade of BK_{Ca} (100nM iberiotoxin (IbTx)) (n = 5). 14,15-EET (n = 3) lacked vasodilator activity in this vessel. *, $p<0.05$; **, $p<0.01$ vs 11,12-EET. The effect of each treatment combination on resting diameter is summarized in Table 1. (C) Time course of relative changes (F/F_0) in endothelial cell $[Ca^{2+}]_i$ in response to 1μM ACh (n = 5), 10μM 11,12-EET (n = 3), and 1μM levcromakalim (LK, n = 5) added at t = 0s. The response to 1μM ACh was also obtained in the presence of raised extracellular K^+ (35mM, n = 3) to inhibit outward K^+ currents and TRAM-34 plus apamin (n = 3). Each n value is the average fluorescence of separate regions placed over 16 entire cells

Treatment	Diameter (% D_{max})	n
Control	46.9 ± 1.2	49
L-NAME	46.8 ± 1.5	32
LN + 35mM K^+	18.8 ± 2.1***	3
LN + Ba^{2+} + Ouabain	40.5 ± 1.6	11
LN + TRAM-34 + Apamin	40.1 ± 2.6	9
LN + IbTx	47.8 ± 2.3	12
LN + 17-ODYA	45.6 ± 3.4	3
LN + MS-PPOH	42.0 ± 3.6	3
LN + TR + AP + IbTx	27.7 ± 2.9***	6
LN + TR + AP + 17-ODYA	32.2 ± 5.4*	3
LN + TR + AP + MS-PPOH	37.1 ± 1.9*	7

Table 5.1; Effect of inhibitor treatments on basal tone. Values are expressed as a percentage of the maximum diameter for each experiment. The average maximum diameter for all experiments was 141.2 ± 3.0 μm (n = 51). *, $p<0.05$; ***, $p<0.001$ compared to L-NAME.

5.3.7 Morphology of rat cremaster arteries

To investigate the possibility that endothelial cell hyperpolarization could be transferred to surrounding smooth muscle via myoendothelial gap junctions, the ultrastructure of arteries was investigated using electron microscopy. Micrographs show that the 1A cremaster arteries studied characteristically had 2 layers of smooth muscle (Figure 5.6), and multiple inter-endothelial cell and myoendothelial gap junctions were evident (Figure 5.6C, D). The myoendothelial junctions showed typical laminar structures indicative of gap junction plaques (Figure 5.6D, inset).

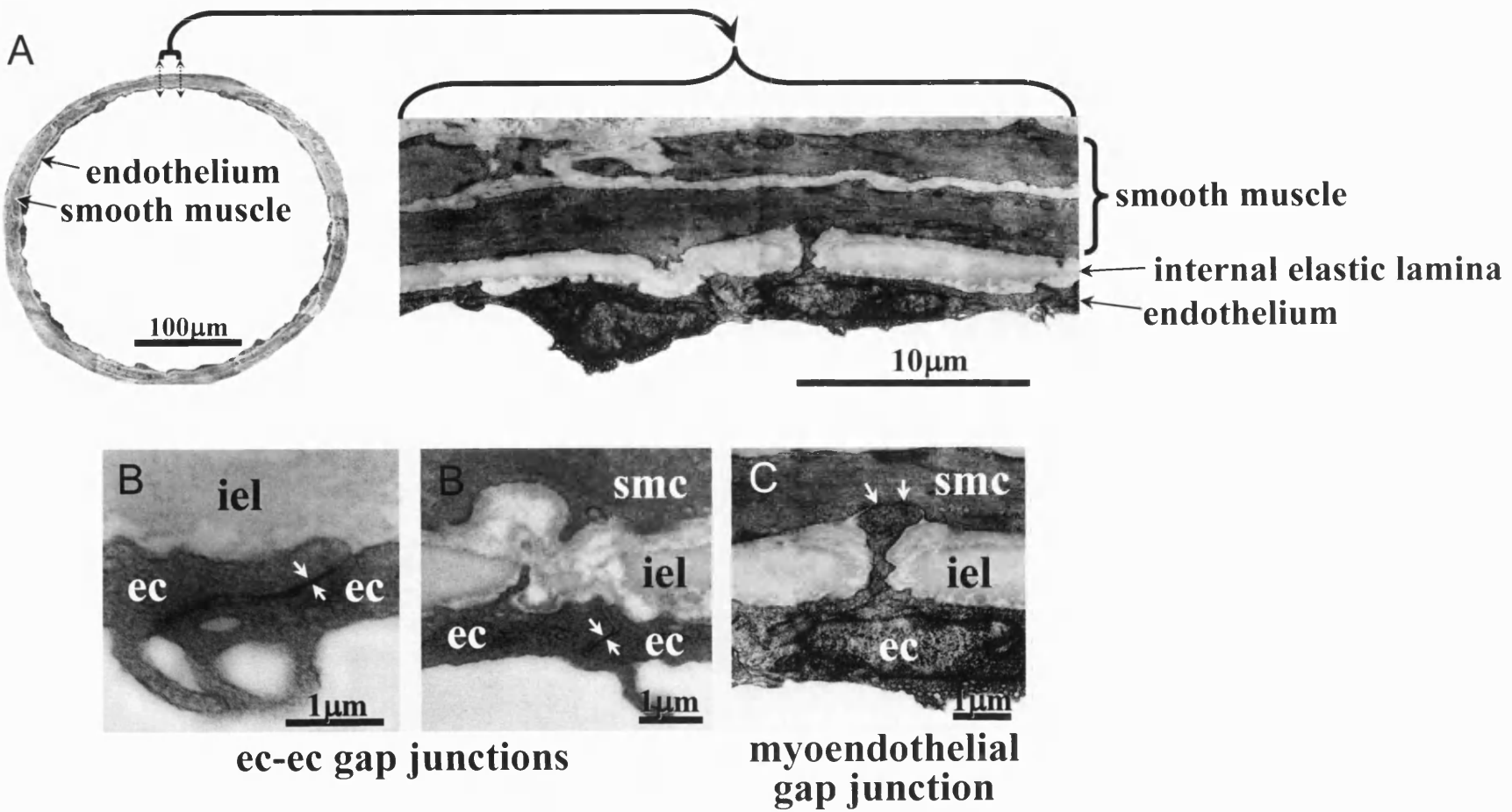

Figure 5.6; Morphology of the rat cremaster artery. (A) Cross-section through a pressurized artery (left) with higher magnification image (right) showing the layers of smooth muscle cells (SMCs), the internal elastic lamina (IEL) and endothelial cell (EC) monolayer. (B, C) Typical EC and myoendothelial gap junctions were observed (arrowheads).

5.4 DISCUSSION

Endothelium-dependent dilatation of myogenically constricted vessels was demonstrated through the use of the endothelium-dependent dilator, ACh. ACh acts on muscarinic receptors on the endothelium that are coupled to G-proteins linked to the formation of IP_3 and DAG. In addition, exogenous ACh has previously been demonstrated to elevate endothelial cell calcium in isolated rat cremaster arteries (Falcone *et al.*, 1993). Both DAG and IP_3 can lead to increased endothelial cell $[Ca^{2+}]_i$, which can then stimulate NO synthase, K_{Ca} channels and increase the formation of CYP-450 metabolites (for review see (Triggle *et al.*, 2003)).

The data demonstrate that ACh stimulates concentration-dependent increases in endothelial cell $[Ca^{2+}]_i$, the range of which closely matched the concentration-dependent dilatation. The intracellular Ca^{2+} waves generated following application of ACh were repetitive, resulting in oscillations in average Ca^{2+} levels within cells, and were similar to those observed in the rat mesenteric artery (McSherry *et al.*, 2005b). However, when responses from 16 cells were averaged, the oscillations in Ca^{2+} were dampened as the asynchronous individual oscillations cancelled each other out. This non-oscillating Ca^{2+} response more closely matches the hyperpolarization recorded from smooth muscle cells (Kotecha *et al.*, 2005), and the steady, uniform vasodilatation. The presence of homo and heterocellular gap junctions supports the possibility of electrical coupling, which would facilitate the artery acting as a functional syncytium. Although evidence for intercellular Ca^{2+} signalling is not provided, the presence of gap junctions at adjacent endothelial cell borders (Looft-Wilson *et al.*, 2004) would theoretically enable this to occur as each Ca^{2+} wave passed along a cell. The possibility for

intercellular Ca^{2+} signalling was reported in mouse cremaster arterioles *in situ* (Duza *et al.*, 2004), where the extent of synchronized spontaneous rises in endothelial cell Ca^{2+} was greater between immediately adjacent cells than randomly selected cells.

Overall, the majority of the dilatation to ACh remained in the presence of the NO synthase inhibitor L-NAME. These L-NAME-insensitive relaxations were fully inhibited by 35mM extracellular K^+, suggesting that the remaining relaxation was due to smooth muscle cell hyperpolarization. Blockade of IK_{Ca} and SK_{Ca} channels in combination revealed that the activation of these channels was in part responsible for the L-NAME resistant dilatation. The increase in endothelial cell $[Ca^{2+}]_i$ would likely stimulate IK_{Ca} and SK_{Ca} channels, these K_{Ca} channels typically found on the endothelium of resistance vessels (Busse *et al.*, 2002). The K^+ efflux through these channels may increase the activity of the electrogenic Na^+/K^+-ATPase and activate inward rectifying K^+ channels (K_{IR}), causing further hyperpolarization (Edwards *et al.*, 1998). Data from the present study showed that elevation of extracellular $[K^+]$, with exogenous isotonic 15mM K^+, stimulated dilatation of cremaster vessels, albeit to a lesser extent than ACh, suggesting K^+ could act as an EDHF, as was observed in the rat hepatic and to a lesser extent, mesenteric arteries (Edwards *et al.*, 1998). Additionally, this dilatation could be inhibited by joint K_{IR} and Na^+/K^+-ATPase inhibition. However, the combined inhibition of K_{IR} and Na^+/K^+-ATPase did not affect ACh mediated dilatation. This lack of effect may reflect insufficient K^+ efflux from endothelial cells, or compensation by the other EDHF pathways. The most likely alternative pathway for endothelium-dependent hyperpolarization of smooth muscle is direct electrical coupling via myoendothelial gap junctions, which are at least structurally evident in rat cremaster arteries. Heterocellular electrical coupling has been

demonstrated in a variety of isolated arteries and arterioles, including hamster cheek pouch (Xia *et al.*, 1995) and retractor muscle feed arteries (Emerson *et al.*, 2000), and rat mesenteric arteries (Sandow *et al.*, 2002), and may account for EDHF responses in these vessels. Definitive studies to confirm this pathway in rat cremaster arteries should involve selective inhibition of myoendothelial gap junction communication. The pharmacological tools available for blocking gap junction communication, such as glycyrrhetinic acid derivatives or connexin mimetic peptides are limited by their specificity (Tare *et al.*, 2002; Mather *et al.*, 2005), however emerging techniques involving intracellularly-targeted antibodies may provide a means to test this hypothesis in the future (Mather *et al.*, 2005).

At higher concentrations of ACh, the L-NAME-insensitive dilatation was not fully inhibited by the combination of TRAM-34 and apamin, suggesting a role for another pathway, which was subsequently found to involve CYP-450 metabolites. Selective blockade of BK_{Ca} with iberiotoxin fully blocked the residual, TRAM-34 and apamin-insensitive, dilatation to ACh, and was matched by inhibition of CYP-450 with 17-ODYA and MS-PPOH. Interestingly, neither iberiotoxin, 17-ODYA, nor MS-PPOH alone had any effect on the L-NAME insensitive component of the response to ACh. Thus, it would appear that the EDHF response is primarily dependent on SK_{Ca} and IK_{Ca} activation, which can account for full dilation even after blockade of the CYP-450 pathway, suggesting that the CYP-450 pathway plays a facilitatory role, or is an 'insurance' vasodilatatory mechanism unmasked ony following SK_{Ca} and IK_{Ca} blockade.

CYP-450 enzyme isoforms have been described in rat cremaster muscle arterioles (Wang *et al.*, 2004), and have previously been demonstrated to produce a variety of vasoactive metabolites (for review see Fleming, 2001). CYP-450 products have also previously been implicated in responses of rat cremaster vessels to ACh (Bakker *et al.*, 1997). Epoxyeicosatrienoic acids (EETs) represent the major products of the CYP-450 epoxygenase, with these compounds and their metabolites possessing dilator activities. Indeed, it has been shown that EETs are released in response to endothelial cell activation, and can exert dilator effects via activation of smooth muscle cell BK_{Ca} channels (Campbell *et al.*, 1996).

Application of exogenous 11,12-EET elicited dilatation, similar in concentration range and magnitude to those reported in other arteries (Campbell *et al.*, 1996; Fang *et al.*, 1996), demonstrating the vasodilatatory actions of a CYP-450 epoxygenase product. The dilatation to 11,12-EET was sensitive to a selective BK_{Ca} channel inhibitor, supporting the possibility that endothelium derived CYP-450 products activate smooth muscle BK_{Ca} channels. Bioassay experiments have demonstrated that released EETs can cause dilatation in detector endothelium-denuded arteries (Dong *et al.*, 1997; Gauthier *et al.*, 2005; Huang *et al.*, 2005), strengthening the argument that released EETs can indeed diffuse to surrounding muscle. Further, it was not possible to rule out an endothelium-dependent component of the CYP-450 metabolite response (Popp *et al.*, 2002; Weston *et al.*, 2005), as in this preparation, the diffusion of exogenously-applied EETs to the endothelium may be compromised.

The lack of any discernible dilatation to 14,15-EET in this preparation was unexpected, as 14,15-EET has previously been described as being a vasodilator of similar potency to 11,12-

EET (Falck *et al.*, 2003). The differences between these isomers in rat cremaster vessels is suggestive of strict structural requirement(s) of the 11,12-EET effectors, which are as yet undefined, but may involve a putative EET receptor (Wong *et al.*, 1993), rather than a more broad mechanism involving, for example, a generic activation of K^+ channels through binding to the pore domain (Ye *et al.*, 2005). The 14,15-EET data further supports control experiments which demonstrate a lack of vehicle effect (0.1% EtOH).

It was not possible to assess whether 11,12-EET facilitated transmission of signals through gap junctions in the intact artery, as reported by others in cultured cells (Popp *et al.*, 2002), but the presence of both inter-endothelial cell and myoendothelial gap junctions in this artery would suggest that an action of released CYP-450 metabolites, including 11,12-EET, on either cell type would likely hyperpolarize both endothelial and smooth muscle cells. Even if this were not the case, the ability of smooth muscle cell hyperpolarization *per se* to evoke a change in endothelial cell Ca^{2+} was investigated by adding 11,12-EET and levcromakalim. Since these agents had no detectable effect, despite stimulating robust dilation, it appears that the endothelium does not amplify vasodilation via this pathway, unless Ca^{2+} events occur in microdomains, perhaps a subplasmalemmal Ca^{2+} control unit (Frieden *et al.*, 2002), which is below the limit of our fluorescence image detection. Unfortunately there are no direct microelectrode measurements of responses to levcromakalim in rat cremaster arteries, although the block of dilatation after inhibition of outward K^+ currents (with 35mM extracellular K^+), suggests the dilatation was dependent on hyperpolarization. Furthermore, the ability for an agonist to stimulate hyperpolarization in association with dilatation is optimal when the artery

is pressurized to 75mmHg (Kotecha *et al.*, 2005), and cromakalim stimulates an outward current in hamster cremaster smooth muscle cells (Jackson, 2000).

A role for endothelial cell hyperpolarization in significantly contributing to maintained Ca^{2+} influx during agonist-induced increases in $[Ca^{2+}]_i$ was also not apparent. The full time course of ACh-stimulated levels of endothelial cell Ca^{2+} remained unaffected following inhibition of outward K^+ currents, or in the presence of TRAM-34 and apamin, consistent with observations in tubes of endothelial cells isolated from hamster cremaster arterioles (Cohen *et al.*, 2005), and in endothelial cells of intact rat cerebral (Marrelli *et al.*, 2003) and mesenteric arteries (McSherry *et al.*, 2005b), but not the rat coronary artery (Knot *et al.*, 1999). The latter may be explained by differences in cell morphology, and/or the expression of Ca^{2+} influx/extrusion pathways or SERCA in this tissue. Despite this, together with the observation that smooth muscle cell hyperpolarization does not affect endothelial cell Ca^{2+}, which was also observed in rat mesenteric arteries (Takano *et al.*, 2004), all available evidence in intact arteries does not support a role for hyperpolarization (of up to ~30mV) in driving Ca^{2+} influx to detectable levels. This is in contrast to studies using endothelial cells in culture, where there is evidence for agonist-mediated membrane potential oscillations, membrane potential driving Ca^{2+} influx to observable levels, and therefore oscillations in endothelial cell Ca^{2+} following (not driving) oscillations in membrane potential (Sage *et al.*, 1989).

In summary, in isolated small cremaster arteries pressurized to 75mmHg, application of ACh results in concentration-dependent increases endothelial cell $[Ca^{2+}]_i$. This rise in Ca^{2+} is associated with activation of both the NO and EDHF pathways, the latter due in part to the

opening of IK_{Ca} and SK_{Ca} channels confined to the endothelium, plus a distinct component associated with opening of smooth muscle BK_{Ca} channels which is only unmasked following IK_{Ca} and SK_{Ca} channel blockade. This latter component is sensitive to inhibitors of CYP-450, and is mimicked by exogenously applied 11,12-EET, suggesting a significant role for this arachidonic acid metabolite as an EDHF in the cremaster artery.

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Chapter Six

Conclusion

The investigations presented within this thesis have probed the nature Ca^{2+} signals arising following endothelial cell activation and the role of the endothelium in vasodilatatory responses, particularly the generation of EDHF.

The results presented herein demonstrate endothelial cell activation, with ACh, resulted in elevation and oscillation of endothelial cell Ca^{2+}. Oscillations in endothelial cell Ca^{2+} were dependent on both a functional intracellular Ca^{2+} store and the entry of extracellular Ca^{2+} following store depletion. Agonist induced endothelial cell oscillations appear to be independent of membrane potential in rat cremaster and rat mesenteric arteries, and whilst undoubted physical coupling exists between endothelial cells, in the form of gap junctions, oscillations remain asynchronous and apparently random. Previous work performed in isolated or cultured endothelial cell preparations demonstrated the membrane potential dependence of Ca^{2+} entry (Schilling, 1989), underlining the dangers of extrapolating results from isolated and cultured cells to a whole vessel physiology.

Increased understanding of the mechanisms controlling endothelial cell Ca^{2+} profiles provide a critical insight into the activation of a cell type which is a key determinant of vessel diameter and thus blood pressure. The findings presented in this thesis have implications for spreading responses, the transfer of vasoactive signals along a vessel wall, which are commonly observed in the vasculature. The endothelium can coordinate vasomotor responses along the length of arteries (Haas *et al.*, 1997; Yamamoto *et al.*, 2001), although it appears that Ca^{2+} influx into endothelial cells is not a major driving force for this process (Takano *et al.*, 2004). The membrane potential independence of Ca^{2+} entry in endothelial cells means that the electrical

spread of hyperpolarization between cells of the arterial wall is a feasible path for the spread of vasodilatatory responses.

Whilst no ubiquitous EDHF mechanism can be described from these studies, the key role of SK_{Ca} and IK_{Ca} channels, presumed to be located on the endothelium, in the transduction of EDHF-like responses was reinforced. Throughout experimentation, combined inhibition of SK_{Ca} and IK_{Ca} channels significantly reduced the EDHF responses studied, and as such likely represent a key event in the generation of EDHF. Heterocellular coupling in the vessel wall, in the form of myoendothelial gap junctions, was also apparent in the vessels studied suggesting an important role for these structures in endothelium derived hyperpolarization of vascular smooth muscle. Previous work has established a correlative link between the presence of myoendothelial gap junctions and endothelium derived hyperpolarization responses (Sandow *et al.*, 2002). Pressurized cremaster arteries exhibited EDHF responses which could be mediated in part through CYP450 products, likely EETs acting at BK_{Ca} expressed on vascular smooth muscle in this vessel. A role for EETs in EDHF responses was only unmasked following NO inhibition and SK_{Ca} and IK_{Ca} blockade, demonstrating a single vessel may possess more than one EDHF mechanism, and emphasising the complex nature of EDHF responses.

Throughout this study and many others the role of EDHF is determined by subtraction, i.e. inhibiting NO and PGI_2 pathways and measuring the remaining relaxations. Whilst allowing the selective investigation of EDHF, this methodology also clouds the precise physiological role of EDHF; is EDHF involved in the physiological control of blood pressure or merely a backup mechanism observed when other pathways have failed? It is a question which is difficult to answer and may in part be dependent on the nature of underlying EDHF

mechanisms. Indeed, CYP450 products are putative EDHFs in certain vessels and the action of CYP450 is inhibited by NO (Wink *et al.*, 1993), indicating interplay between the two systems and perhaps a predominant role for NO. However, in rat middle cerebral arteries EDHF responses are not suppressed by NO (Schildmeyer & Bryan, 2002) suggesting a role for EDHF during normal physiological conditions. Less ambiguous are the alteration of EDHF responses in various pathophysiological conditions, including ageing, hypertension, atherosclerosis, and hypercholesterolemia (for review see Feletou & VanHoutte, 2004). Furthermore, certain therapeutic interventions, such as oestrogen or polyphenol derivatives, have also been demonstrated to restore or enhance EDHF responses (for review see Feletou & VanHoutte, 2004). Increasing the activation, and/or the expression, of endothelial K_{Ca}, or the selective increase of endothelial cell Ca^{2+} may represent interesting therapeutic possibilities. The apparent non-ubiquitous mechanisms of endothelium-derived hyperpolarization of vascular smooth muscle could also represent an oppurtunity to modulate EDHF responses in a specific population of vessels, allowing for the selective targeting of therapeutics.

Chapter Seven

Future directions

Transmural pressure in the control of endothelial Ca^{2+}

Recent evidence from an isolated perfused juxtamedullary nephron preparation demonstrated a decrease in endothelial cell Ca^{2+} with increases in perfusion pressure (Pittner *et al.*, 2005). It remains possible that the physiological 'switching off' of the endothelium at high pressures may be a mechanism where vasodilatory influences from the endothelium are reduced to allow myogenic tone, an important vasoregulatory mechanism to develop. Supporting this hypothesis in the cremaster the endothelium independence of the myogenic response generating mechanisms has been reported ((Falcone *et al.*, 1991) and unpublished observations). If this hypothesis were to be true, the endothelium would still be able to exert vasodilatory influences through shear stress induced release of mediators which are independent of rises in endothelial intracellular Ca^{2+} (for review see (Busse *et al.*, 2003)). Preliminary experiments have revealed a similar effect in isolated rat cremaster arteries. In arteries pressurized to 75mmHg basal oscillations decrease in frequency and amplitude over time, whereas in arteries pressurized to 15mmHg endothelial cell Ca^{2+} continues to oscillate (see figures 7.3 and 7.4). Furthermore, a pressure step from 15 to to 75 mmHg leads to a decrease in oscillations in and levels of endothelial cell Ca^{2+}, whilst the reciprocal Ca^{2+} response occurs when the pressure step is made in the opposite direction (see figures 7.1 and 7.2). This effect is of potentially critical importance in the maintenance and generation of myogenic tone and could represent an important development in the understanding of physiological endothelial activation and deactivation.

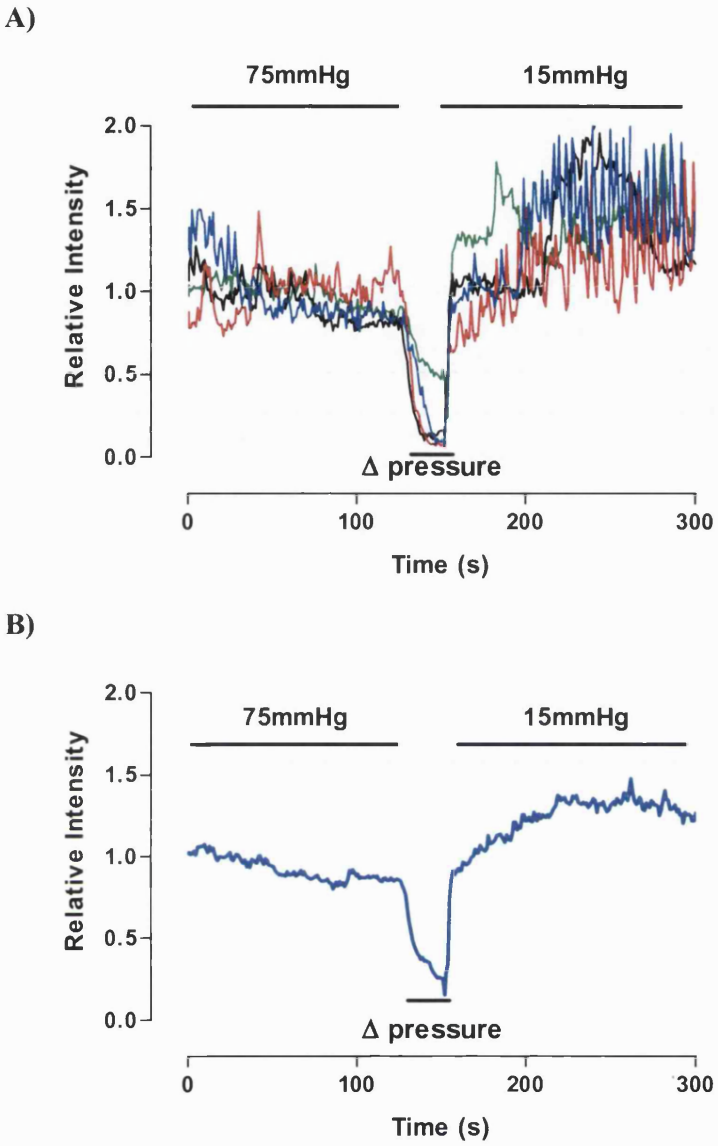


Figure 7.1; Representative traces detailing the effect of reducing transmural pressure on endothelial cell Ca^{2+} in pressurised rat cremaster arteries. A) Each line represents the recording of the fluorescence intensity of a single endothelial cell, vessels are subjected to a pressure step from 75mmHg to 15mmHg. elevation in endothelial cell Ca^{2+} is seen with a concommitant increase in endothelial cell Ca^{2+} oscillations. B) averaged data presented in A).

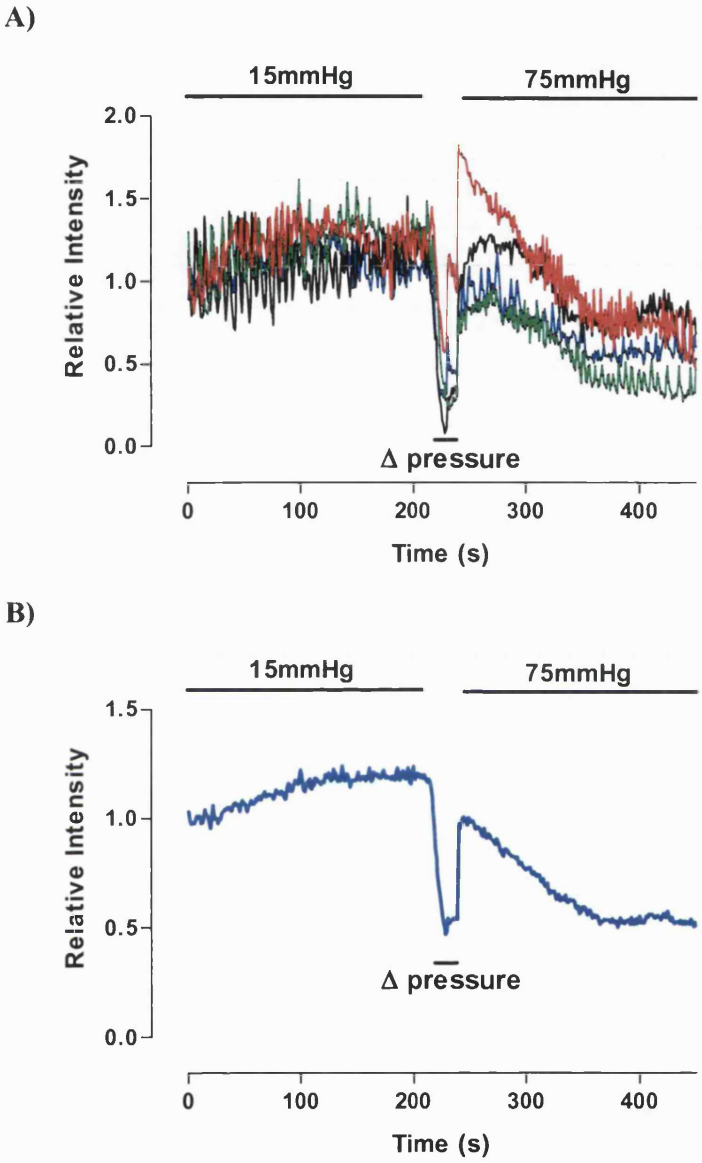


Figure 7.2; Representative traces detailing the effect of elevating transmural pressure on endothelial cell Ca^{2+} in pressurised rat cremaster arteries. A) Each line represents the recording of the fluorescence intensity of a single endothelial cell, vessels are subjected to a pressure step from 15mmHg to 75mmHg. Reduction in endothelial cell Ca^{2+} is seen with a concommitant reduction in endothelial cell Ca^{2+} oscillations. B) averaged data presented in A).

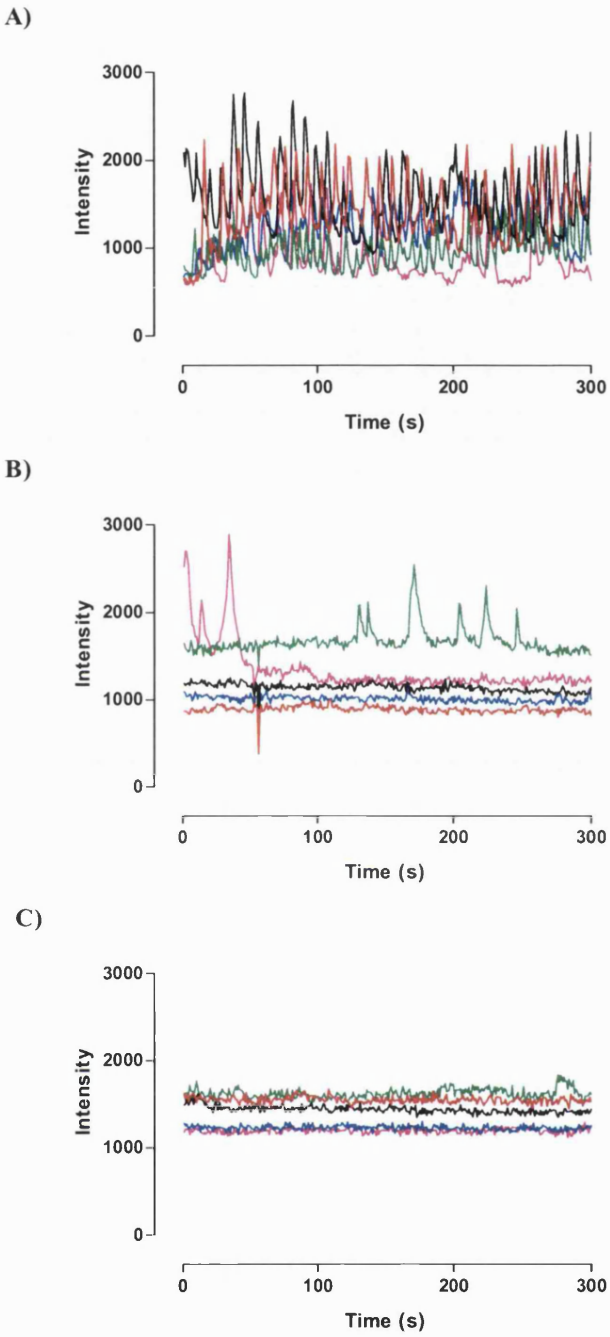
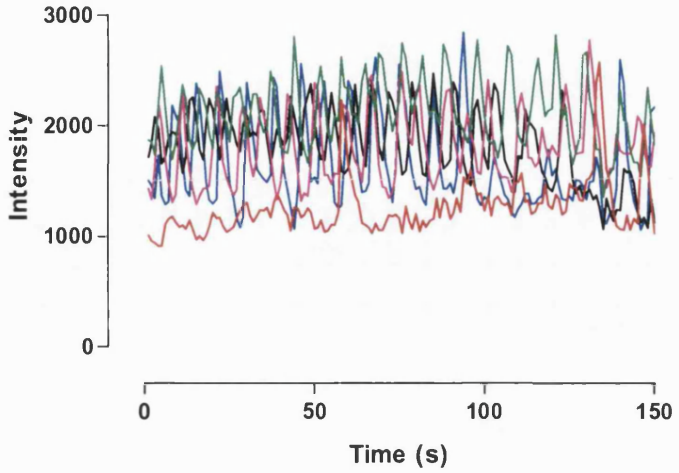


Figure 7.3; Representative traces detailing endothelial cell basal oscillations in Ca^{2+} in pressurized rat cremaster arteries at 75mmHg. Each line represents the recording of the fluorescence intensity of a single endothelial cell. A) Following a dye loading period of approximately 30mins at 10mmHg, vessels are pressurised to 75mmHg, with nearly all endothelial cells oscillating over a 200s time-course. B) 15mins following the pressure elevation to 75mmHg, the oscillating cells are reduced in number and the frequency of oscillation is less in those cells which do oscillate. C) 30mins following pressurisation, minimal-to-no basal oscillations are apparent in endothelial cell Ca^{2+}.

A)



B)

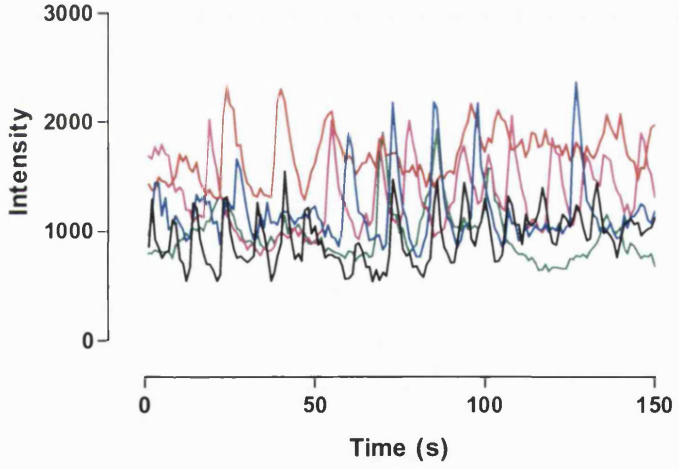


Figure 7.4; Representative traces detailing endothelial cell basal oscillations in Ca^{2+} in pressurized rat cremaster arteries at 15mmHg. Each line representing a recording of the fluorescence intensity of a single endothelial cell. A) Following dye loading, endothelial cell oscillations at 15mmHg were monitored, with nearly all endothelial cells oscillating over a 150 second time-course. B) 50 minutes following dye loading at a constant transmural pressure of 15mmHg endothelial cells still elicit spontaneous oscillations.

The authors of the report in the isolated perfused juxtamedullary nephron speculate that, as the release of shear stress mediated NO is increased when the pressure is increased, there may be a feedback control of this nitric oxide on endothelial Ca^{2+} (Pittner *et al.*, 2005). However there may be a simpler explanation. It is feasible that the increasing depolarization of vascular smooth muscle observed with increasing transmural pressures (Harder, 1984; Kotecha *et al.*, 2005) may transfer to endothelial cells via myoendothelial gap junctions, and simply decrease Ca^{2+} entry by reducing the electrochemical gradient for Ca^{2+} entry. Whilst emerging evidence would suggest that agonist induced endothelial Ca^{2+} entry is membrane potential independent in whole vessel preparations (Cohen *et al.*, 2005; McSherry *et al.*, 2005a; McSherry *et al.*, 2005b), this may not be the case for non-agonist induced Ca^{2+} entry. Given the vital autoregulatory role of both the myogenic response and the endothelium it is vital to understand their physiology, thus warranting further investigation.

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